Dedicated to the loving memory of my Grandmother,

Esther Fenwick.

# ACKNOWLEDGEMENTS

I would like to thank Dr. Paul Rooney for all of his invaluable supervision, help and support in all aspects of this project. Mr. Mike Roberts for his help in a number of technical matters, the photography and for his general support and encouragement, Miss Anne-Marie van Zeller for her help, support and encouragement throughout, Dr. Steve Chappell and Dr. Tom Walsh from the Breast Cancer Research Unit at Leicester University for their invaluable help with the RT-PCR methods, Prof. Paul Gregg, Mr. Joe Harper, Mr. Olusola Oni, Mr. Jon Davison and Mr. Al Best for the provision of human articular cartilage and Miss Louise Reeve for her encouragement and support for the last few months. Thanks also to the many un-named others within the University departments of Orthopaedic Surgery, Respiratory Medicine, Cardiology, Chemical Pathology, the Breast Cancer Research Unit and also the Leicester Bone Bank for all the help and the massive support that they have given me over the past three years. Finally, thanks to my parents for their continued support in everything I have done throughout my time here.

# Models of Cartilage Vascularisation: The Vasculature and its Effect on Developing and Osteoarthritic Cartilage.

**Thesis submitted for the degree of Ph.D.**
**at the University of Leicester**

by

S.A. Fenwick B.Sc. (Newcastle-Upon-Tyne)

Department of Orthopaedic Surgery

University of Leicester

October 1997.





ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

# Models of Cartilage Vascularisation: The Vasculature and its Effect on Developing and Osteoarthritic Cartilage.

S. A. Fenwick, 1997.

## ABSTRACT

The vascularisation of cartilage, despite its importance in development and a number of disease processes has yet to be fully elucidated. The work presented here lends evidence for a major role for the vasculature in the erosion of cartilage. A soluble factor or factors produced by the endothelium is responsible for the degeneration and subsequent death of hypertrophic chondrocytes. The cartilage itself, however, may also have a role, as it is also shown that only a particular subset of hypertrophic chondrocytes can become invaded by the vasculature, and only at a specific time when placed on the chick chorio-allantoic membrane (CAM). Evidence is provided that the control of this may be periosteally derived, and further evidence suggests that the breakdown of cartilage hyaluronan may be an initiating factor in the process.

The collagen expression of the degenerate chondrocytes is not adversely affected qualitatively by the endothelium, except for a loss of type X collagen, though quantitatively, there is a large reduction in the amount of collagen produced. Of importance, is that throughout the degeneration process, and despite the loss of chondrocyte morphology, the cells maintain expression of type II collagen and there is no specific switch to type I collagen production that is associated with some morphological changes in chondrocytes. The ultimate fate of these chondrocytes could not be convincingly elucidated.

Finally, the vascularisation of human OA cartilage was studied. Human OA cartilage loses its ability to remain avascular when placed into an *in-vivo* model of vascularisation, the chick CAM. The vascular invasion is associated with a loss of histochemical staining for proteoglycans and glycosaminoglycans and a deposition of type I and type X collagens around the invasive vessel.

# PUBLICATIONS & ABSTRACTS

Publications:

Fenwick S. A., Gregg P. J., Kumar S., Smith J. & Rooney P. (1997) Intrinsic Control of Vascularisation in Developing Cartilage Rudiments. *Int. J. Expt. Pathol.* **78**, 187-196.

Abstracts:

Fenwick S. A., Gregg P. J. & Rooney P. (1995) Is there a Temporal and/or Spatial Switch to an Angiogenic Phenotype During Cartilage Neovascularisation? *Int. J. Expt. Pathol.* **76**, A14.

Fenwick S. A., Gregg P. & Rooney P. (1996) The Loss of Avascularity in Human Osteoarthritic Cartilage. *Int. J. Expt. Pathol.* **77**, A5.

Fenwick S. A., Gregg P. & Rooney P. (1996) An *In-Vitro* Model of Cartilage Angiogenesis. *J. Bone Joint Surg.* **78-B SUPP I,** 37.

Fenwick S. A., Gregg P. & Rooney P. (1997) The Loss of Avascularity in Human Osteoarthritic Cartilage. *J. Bone Joint Surg.* **79-B: SUPP III,** 363.

# Contents:

# TABLE CONTENTS

# FIGURE CONTENTS

CHAPTER 4:



CHAPTER 5:



CHAPTER 6:

# CHAPTER 1

# Introduction

# 1. INTRODUCTION

## 1.1 Cartilage Formation

### 1.1.1 The Origins of Limb Cartilage:

In the developing embryo, cartilage can have a variety of forms from simple sheets or rods, to complex 3-dimensional structures like vertebrae (Archer *et al.*, 1983). Cartilage cells, known as chondrocytes, along with all other connective tissues in the developing limb, derive from mesenchymal cells (Solursh *et al.*, 1982; Thorogood, 1983; Solursh, 1989). The development of cartilage from this primitive mesenchyme begins with the condensation of these mesenchymal cells (Thorogood & Hinchcliffe, 1975; Archer *et al.*, 1983; Hallmann *et al.*, 1987; Price *et al.*, 1994). These condensations mark the position of the future skeletal elements and occur prior to overt chondrogenesis (Archer *et al.*, 1983). The differentiation of the cells into chondrocytes proceeds in a temporally and spatially defined manner, the factors controlling this, however, are unknown (Hallmann *et al.*, 1987). Chondrogenesis involves a combination of cellular division, growth, matrix secretion, specifically type II collagen and sulphated glycosaminoglycans (GAG) (Archer *et al.*, 1982), and specific cell matrix interactions (Miller & Matukas, 1969; Solursh *et al.*, 1982; Archer *et al.*, 1984; Horton & Hassell, 1986). The developing cartilage is not, however, a simple mass of chondrocytes, and throughout its development, is surrounded by other cell types which are non-chondrogenic. Interactions between these and the developing cartilage are likely to occur. In fact, cartilage morphogenesis *in-vivo*

is thought of as a product of a series of interactions between cells, between cells and matrix and between tissues (Archer *et al.*, 1984; Rooney *et al.*, 1984).

The tissue in direct contact with the cartilage is the fibrous perichondrium. This has been argued to play an early and crucial role in the morphogenesis of the long bone rudiment in that its central portion becomes a restraining sheath, favouring longitudinal growth of the cartilage, termed directed dilation (Wolpert, 1982; Archer *et al.*, 1983), while the extremities of the perichondrium are weaker and do allow some expansion of the enclosed cartilage (Archer *et al.*, 1983; Rooney *et al.*, 1984; Rooney & Archer, 1992). There appears, then, to be a mechanical control involved in the morphogenesis of the long bone rudiment.

Chondrogenesis is initiated in the central region of the rudiment and subsequently spreads towards the ends (Fell, 1925). The first cells to secrete matrix become flattened perpendicular to the long axis of the limb (Archer *et al.*, 1983; Rooney *et al.*, 1984). At about the same time, cells around this central cartilage cylinder flatten parallel to the long axis to form a rudimentary perichondrium (Gould *et al.*, 1974; Archer *et al.*, 1983; Rooney & Archer, 1992). Within a day of the start of chondrogenesis, cells within the rudiment form five cellular zones along the long axis (Fell, 1925), each with a distinct cellular morphology- rounded, flattened, hypertrophic, flattened, rounded (Figure 1.1).

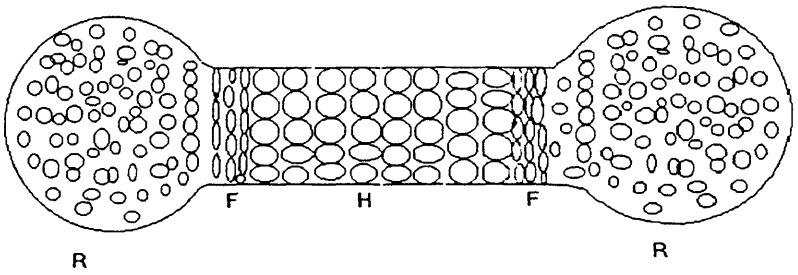



**Figure 1.1.** The developing cartilage rudiment showing the five distinct zones of cells, H is the hypertrophic cell region, F is the flattened cell region, R is the rounded cell region.

### 1.1.2 The Cartilage Rudiment:

The development of cartilage long bone rudiments in both avian and mammalian systems, and their subsequent replacement by bone share a number of identical steps (Roach & Shearer, 1989: Caplan, 1990). Both systems have been studied extensively as models of bone formation (Fell, 1925; Silvestrini *et al.*, 1979; Reddi & Kuettner, 1981; Pechak *et al.*, 1986a & b; Roach & Shearer, 1989; DeSimone & Reddi, 1992; Lewison & Silbermann, 1992; Galotto *et al.*, 1994). However, there are distinct differences, most notably that developing avian bones do not contain a distinct growth plate, the cartilage matrix does not calcify prior to resorption (Roach & Shearer, 1989) and the cartilage is replaced by marrow and not bone (Fell, 1925).

The continuing morphogenesis of the cartilage rudiment occurs through growth. This can be viewed as three distinct processes - cell division, extracellular matrix (ECM) secretion and cell hypertrophy (Archer *et al.*, 1983; Thorogood, 1983; Rooney & Archer, 1992). Cell hypertrophy begins in the centre of a rudiment, where the cells enlarge to nearly three times their original diameter and withdraw from the cell cycle (Fell, 1925; Archer & Rooney, 1984). Surrounding lacunae are also seen to be enlarged to accommodate the increase in cell volume (Schmid *et al.*, 1990). Chondrocyte hypertrophy is believed to be an autonomous process (Archer *et al.*, 1984) and appears to be a distinct differentiative state, since cells maintain a hypertrophic morphology and behaviour in cell culture (Rooney, 1984). This cellular hypertrophy extends into

the flattened cell zone where cells gradually lose their compressed appearance, becoming vacuolated and distended (Fell, 1925). The rounded cell zones now appear very distinct.

Pre-hypertrophic chondrocytes, and non-hypertrophic chondrocytes, synthesise a mixture of type II, IX and XI collagens which co-assemble into heterotypic fibrils (Mayne, 1989; Mendler *et al.*, 1989; Schmid *et al.*, 1990; Trippel, 1990; Kuettner 1992; Sandell *et al.*, 1994). They also synthesise the large aggregating proteoglycan, aggrecan (Bonaventure *et al.*, 1994; Sandell *et al.*, 1994). Once the cells become hypertrophic, they also secrete type X collagen, whose synthesis increases along with a concomitant decrease in synthesis of the others as the hypertrophic program progresses (Leboy *et al.*, 1989; Schmid *et al.*, 1990; Sullivan *et al.*, 1994).

The ECM of the central zone is very dense due to compression by secretion of additional matrix and by the enlargement of the cells (Fell, 1925). The rounded cells lie in a close regular network, the collagen oriented in a random fashion (Archer *et al.*, 1984; Rooney *et al.*, 1984). The collagen of the flattened zones is oriented parallel to the planar surface of the cells and appears mat-like in transverse sections (Archer *et al.*, 1984; Rooney *et al.*, 1984). This matrix is at first looser than that of the hypertrophic zone, but eventually becomes equally as dense (Fell, 1925).

The life cycle of the chondrocyte, therefore, has a number of stages; a proliferative stage and a hypertrophic stage where the cells are biosynthetically

active, and finally a degenerative stage (Kirsch & von der Mark, 1991). The degenerative stage is seen as a condensation of the hypertrophic chondrocytes (Fell, 1925), thought to indicate necrosis (Roach & Shearer, 1989) and coincides with the removal of the chondrocytes during the cartilage invasion and marrow formation, which make up part of the primary osteogenic events of bone formation. The reason for the degeneration, and in fact, the actual fate of the chondrocyte, is not fully understood, and several theories exist about their ultimate fate (Silvestrini *et al.*, 1979; Lewinson & Silbermann, 1992; Gibson *et al.*, 1995a)

### 1.1.3 Bone and Articular Cartilage:

Cartilage, bone and bone marrow are the three main components involved in long bone formation (Cole *et al.*, 1992). Bone formation is known to occur via two distinct pathways. Endochondral ossification is the replacement with bone of a hyaline cartilage model. Intramembranous ossification is the direct differentiation of mesenchymal tissue into bone with no cartilage intermediate (Scott & Hightower, 1991). Mammalian long bones develop by endochondral ossification, the majority of the cartilage model being replaced by bone except for a layer between the diaphysis and epiphyses - the growth plate, and a thin covering at the ends of the bone - the articular cartilage.

As the process of chondrocyte hypertrophy continues in mammals, major changes occur in the ECM of the cartilage. Calcification of the ECM occurs

and this provides a substratum onto which bone can be deposited (Schmid *et al.*, 1990). In avians, mineralisation of the cartilage does not generally occur (Roach & Shearer, 1989) and there is a lack of true endochondral ossification, except at the extremities of the diaphysis during the late stages of development (Fell, 1925).

The initiation of osteogenesis occurs outside of the central cartilage core (Pechak *et al.*, 1986b) as a result of inner perichondrial cells differentiating into bone forming cells (osteoblasts) within what is now termed the periosteum (Price *et al.*, 1994). The first bone matrix is therefore of periosteal origin, and forms a continuous collar of osteoid around the cartilage core. This osteoid subsequently becomes mineralised (Pechak *et al.*, 1986b; Roach & Shearer, 1989). Replacement of the cartilage core by bone requires invasion by the vasculature present in the periosteum (Reddi & Kuettner, 1981; Pechak *et al.*, 1986b; Roach & Shearer, 1989; DeSimone & Reddi, 1992; Price *et al.*, 1994), forming the primary or diaphyseal ossification centre. In avian systems, vascularisation still proceeds through the periosteum into the hypertrophic cartilage, though the cartilage matrix is not calcified (Fell, 1925). Shortly after this primary invasion, a separate invasion occurs in the epiphyses, of both mammalian and avian systems, (Fell, 1925; Price *et al.*, 1994) to form the secondary centre of ossification.

Cartilage around the primary ossification centre in mammals is eroded by the vasculature to form the marrow cavity (Pechak *et al.*, 1986b; Roach & Shearer, 1989). New trabecular bone is deposited onto the cartilaginous

remnants that are gradually resorbed to form the medullary cavity (Price *et al.*, 1994). Continued growth of the embryonic bone occurs by appositional growth to increase the width (Roach & Shearer. 1989; Price *et al.*, 1994) and by expansion of the epiphyses to increase its longitudinal size (Roach & Shearer, 1989).

The secondary ossification centre within the epiphyses erodes most of the cartilage at the growing ends. Separating the primary and secondary ossification centres is a transverse plate of cartilage, the epiphyseal growth plate, extending across the bone, which persists until growth of the bone ceases. The cartilage here grows continually but does not thicken, as on the diaphyseal side, the cartilage becomes calcified, resorbed and replaced by bone as part of the endochondral ossification process (Price *et al.*, 1994). This continuous growth and resorption is responsible for the longitudinal growth of bones post birth. Increases in diameter are achieved by new bone laid down by the osteogenic layer of the periosteum (Price *et al.*, 1994). The secondary ossification centre also leaves behind a thin layer of cartilage at the articular ends of the bones, which develops into the articular cartilage.

## 1.2. Articular Cartilage:

Articular cartilage is a highly organised. extremely specialised connective tissue. Its function is to facilitate load bearing in the diarthroidal joints by transmitting the load across the joint. cushioning the underlying subchondral

bone. It also provides an extremely low resistance during motion, allowing free movement of the joint (Jeffery, 1994). Articular cartilage is highly resistant to compression due to its special physical and mechanical properties. These properties are based on the chemical composition and metabolic activity of the tissue (Kuettner, 1992). The composition of articular cartilage is almost entirely ECM, into which is embedded chondrocytes, generally consisting of less than 5% of the tissue by volume (Kuettner, 1992). The matrix consists mainly of a proteoglycan gel and water reinforced by collagen fibres and a number of non-collagenous, non-proteoglycan proteins which include chondrocalcin (the C-terminal polypeptide of type II collagen), anchorin, cartilage oligomeric matrix protein, thrombospondin, fibromodulin and traces of fibronectin (Heinegard & Oldberg, 1989; Kuettner, 1992).

Collagen is the most prevalent organic component of articular cartilage, providing the tensile strength of the tissue, comprising approximately 50-60% of the dry weight or 10-20% of its wet weight (Lane & Weiss, 1975; Trippel 1990; Kuettner, 1992; Jeffery, 1994). Approximately 30% of the dry weight is proteoglycan in nature, the rest being made up of glycoproteins, lipids and chondrocytes (Jeffery, 1994).

90% of the collagen in adult articular cartilage is type II collagen, the rest being composed primarily of types VI, IX, XI (Trippel, 1990; Kuettner, 1992). The collagens are organised into fibrils and form a network onto which the other cartilage components are laid.

Articular cartilage is not homogeneous in terms of its chemical

composition, organisation and chondrocyte morphology throughout its depth, and as such, distinct zones can be described (Lane & Weiss, 1975; Jeffery, 1994). The superficial (or tangential) zone at the articular surface is the most cellular region, the cells here are small and disc shaped (Poole *et al.*, 1993). The ECM is composed of fine collagen fibrils orientated parallel to the surface (Lane & Weiss, 1975). The concentration of proteoglycans here is relatively low (Schumacher *et al.,* 1994). The next zone down, the intermediate (or transitional) zone has more spheroidal and randomly distributed cells (Jeffery, 1994). The ECM here has a higher concentration of proteoglycan and the collagen fibrils are larger in diameter and acquire a more perpendicular orientation (Schumacher *et al.,* 1994) though are more randomly arranged (Lane & Weiss, 1975). The ECM in the third or radiate (deep) zone is similar to the intermediate zone, though the fibres are thicker (Lane & Weiss, 1975), the cells being arranged into a more columnar fashion perpendicular to the surface (Jeffery, 1994; Schumacher *et al.,* 1994). Finally there is a layer of calcified ECM lying adjacent to the subchondral bone. Additionally, at the very surface of the cartilage is a thin, acellular layer composed of collagen fibrils, loosely connected to the superficial layer (Figure 1.2). This was first reported by MacConaill (1951) and termed the 'lamina splendens'. Though this was later reported to be an artefact by Aspden & Hukins (1979), it was finally confirmed to exist by Dunham *et al.,* (1988). This outer coating is anchored to the synovial tissue, its orientation tending to resist tensile stress during movement (Teshima *et al.,* 1995). As a functional entity, it is thought to maintain the morphological

integrity of the cartilage by withstanding extrinsic compression and intrinsic swelling pressure (Teshima *et al.*, 1995).

Several models have been proposed for the overall organisation of articular cartilage. The earliest came from Benninghoff (1925), using polarised light microscopy, who proposed an arcade model whereby the collagen fibres arise in the subchondral bone, pass to the surface in a radial manner before arching to run tangentially to the surface and then return to the subchondral bone. A similar model was proposed by Clark (1985) using SEM. However, a combination of polarised light and phase contrast microscopy led MacConnaill (1951) to propose that the collagen fibres form a dense network running obliquely between the articular surface and the subchondral bone. More recently, SEM and freeze fracture techniques (Jeffery *et al.*, 1991) have led to the proposal of a layered or leaf like structure of collagen fibrils that lie parallel to the surface in the superficial zone and curve into the deeper zones to become more vertical.

The meshwork of collagen fibrils forms compartments in which the proteoglycan complexes are compressed to about one-fifth their maximal volume observed in free solution (Jeffery, 1994). Proteoglycans are highly hydrophilic and this gives articular cartilage a high osmotic pressure but, due to their compression in the matrix, they are in an underhydrated form (Kuettner, 1992). Their attraction for water gives them a tendency to swell, which means

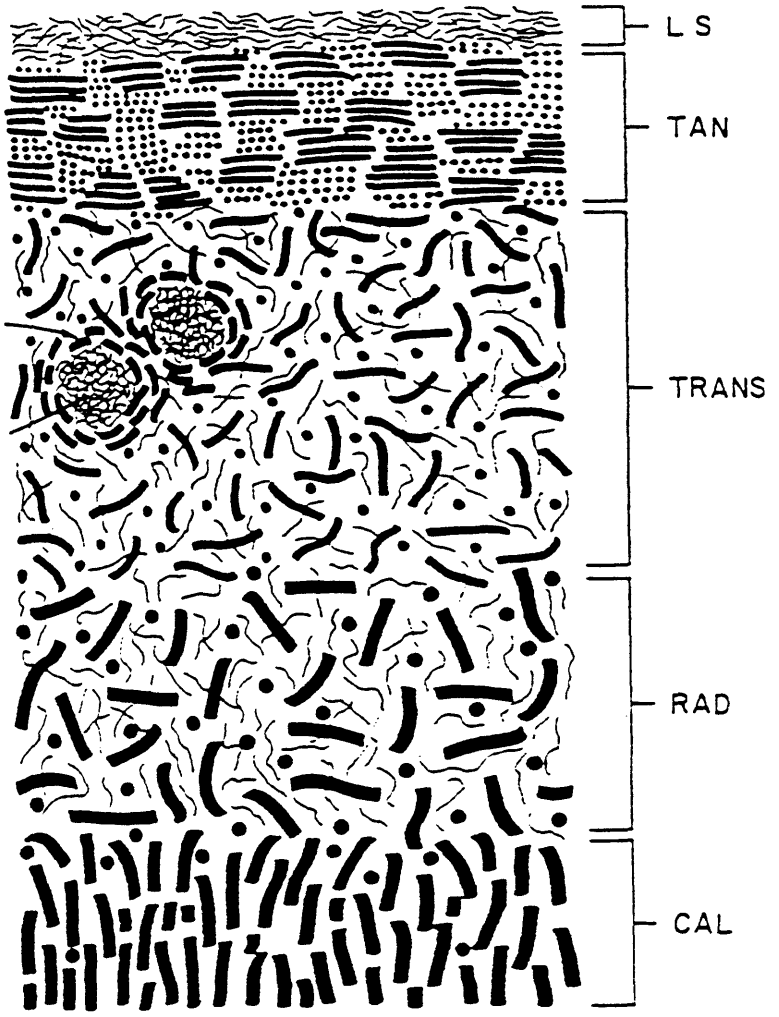



**Figure 1.2.** Diagrammatic representation of the collagen architecture of articular cartilage. LS is the lamina splendens, composed of fine fibres. TAN is the tangential zone, consisting of tightly packed bundles of individual collagen fibres lying parallel to the surface. The transitional zone (TRANS) is composed of thicker fibres which are randomly arranged, amongst which are finer fibres. Fibres in the radial zone (RAD) are also arranged randomly and contain even thicker fibres, below which is the calcified zone (CAL) which contains mature collagen fibres arranged perpendicular to the articular surface. [from (Lane & Weiss, 1975)]

that the collagen network is always under pressure to resist this swelling and as such is always under a certain amount of stress (Jeffery, 1994).

Articular cartilage has long been considered a unique tissue due to its paucity of nerves, lymphatics and vascular tissue (Mankin, 1974a; Hamerman 1984; Kuettner 1992). The lack of nerves is likely to mean that superficial damage to the cartilage is not detected, and only damage that penetrates to the subchondral bone will result in nervous stimulation and pain perception. The lack of a blood and lymphatic system means that chondrocyte nutrition and removal of their waste products rely on diffusion through the cartilage matrix to and from the synovial fluid (Mankin *et al*, 1974a). Chondrocytes also lack direct cell-cell contact (Kuettner, 1992; Bonaventure *et al.*, 1994), therefore communication between cells must also occur via the ECM. There are, however, suggestions that chondrocytes may after all have some form of cell-cell contact (P. Rooney, personal communication).

## 1.3. The Components of Articular Cartilage:

### 1.3.1 Chondrocytes:

Chondrocytes are the cellular component of cartilage and are responsible for synthesising, maintaining and assembling their surrounding matrix (Kuettner, 1992; Jeffery, 1994). Fully differentiated chondrocytes are morphologically rounded or polygonal with scalloped borders, and contain an extensive rough endoplasmic reticulum network, large golgi complexes and a number of

secretory vacuoles (Kosher, 1983). The main distinguishing biochemical features of chondrocytes, compared to their pre-cartilaginous mesenchymal precursors, are their ability to synthesise type II collagen and a cartilage specific sulphated proteoglycan. This proteoglycan differs from non-cartilage sulphated proteoglycans in terms of its protein core. length of chondroitin sulphate (CS) (smaller) and keratan sulphate (KS) (larger) chains and ratio of the 4 to 6-sulphated CS molecules (Kosher, 1983).

Chondrocyte origin and development have been briefly discussed earlier. Postnatally, chondrocytes remain present in cartilage throughout life. In adult articular cartilage, chondrocytes occupy approximately 5% of the total volume, and though they are metabolically active. they do not normally divide after adolescence (Kuettner, 1992), and are believed to maintain their biosynthetic and morphological features throughout life (Treadwell & Mankin, 1986). This however does raise a question in that are chondrocytes in older people truly several decades old??

As mentioned previously, articular cartilage can be classified into several distinct but merging zones showing differing morphologies of the chondrocytes. The superficial layer houses discoidal chondrocytes, their long axes lying parallel to the articular surface. Immediately below, in the intermediate layer, the cells are more spheroidal and are randomly distributed. The deep layer contains chondrocytes that appear arranged as columns at right angles to the joint surface (Figure 1.3). There is also zonal variation in the metabolism of the chondrocytes, reflected in the varying biochemical composition of the cartilage

in the different zones (Kuettner, 1992).

Articular chondrocytes live in an anoxic environment and carry out their metabolism through mostly anaerobic pathways. The possible lack of cell-cell contact means that communication occurs via the ECM, and the delivery of nutrients to and removal of waste from the cells must occur via diffusion through the ECM (Kuettner, 1992). Individually, each chondrocyte is responsible for the synthesis, construction and maintenance of its surrounding ECM, which in itself can be compartmentalised. The matrix encircling each chondrocyte is termed the territorial matrix (Dijkgraaf *et al.*, 1995; Soames, 1995), and is very rich in proteoglycans with a lack of fibrillar collagen (Dijkgraaf *et al.*, 1995). Surrounding this is an area composed of a network of cross-linked fibrillar collagens encapsulating the cell. This is referred to as the chondron, and is regarded as the functional unit of articular cartilage (Gardner, 1994). Most, if not all of the type VI collagen is located here (Poole *et al.*, 1988). The largest, and furthest removed compartment of the cartilage ECM is the interterritorial matrix which contains most of the proteoglycans and collagen fibrils (Soames, 1995). This compartment appears metabolically more inert than the closer compartments (Kuettner, 1992).

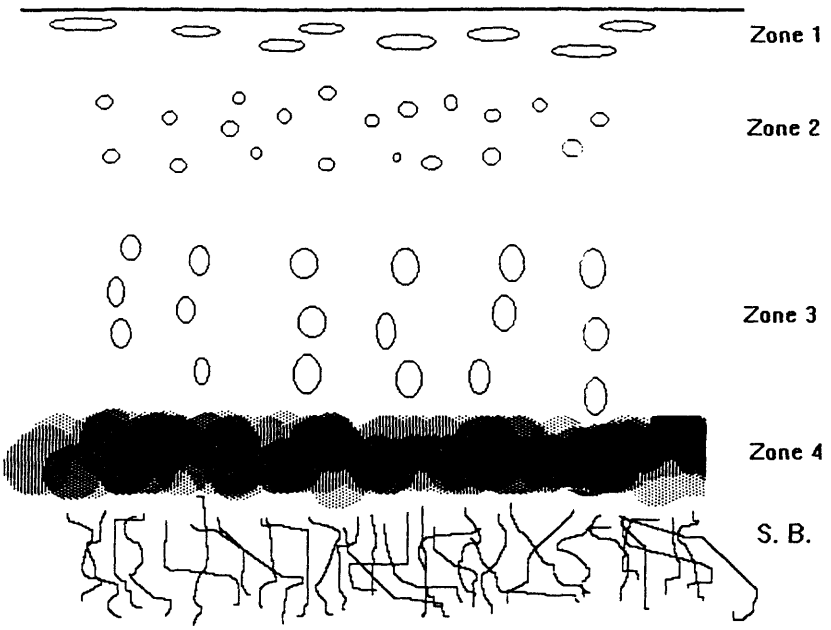



**Figure 1.3.** Diagrammatic representation of the chondrocytes in articular cartilage. Zone 1 shows the flattened disc shaped cells near the surface, zone 2 is the small rounded cells, zone 3 shows large columnarly organised cells and zone 4 is the region of calcified cartilage. S. B. is the subchondral bone [adapted from Jeffery, 1994]

### 1.3.2 Collagen:

Collagen is the single most abundant protein in mammals (Burgeson & Nimni, 1992). Until the end of the 1960's, only one collagen type was known to exist. However, in 1969, via the examination of cyanogen bromide extracts, Miller & Matukas (1969) discovered that the collagen derived from chick cartilage had a primary structure distinct from the collagen isolated from skin and tendon. This new collagen was subsequently named type II collagen. There are now at least 19 collagen types discovered across a variety of tissues, and many more proteins exist that are candidates to be classed as collagens (Brown & Timpl, 1995), which contain the Gly-X-Y repeat (see below). These include complement component C1q, serum mannose binding protein and pulmonary surfactant apoprotein SP-A (Linsenmayer, 1991). Cartilage is known to contain at least 5 genetically distinct collagens, types II, VI, IX, X and XI (Burgeson & Hollister, 1979; Trippel, 1990; Eyre, 1991; Kuettner, 1992).

The basic structure, at the amino acid level, of a collagen molecule is 3 polypeptide chains, known as α chains, coiled around each other to form a triple helix. Each chain consists mostly of Gly-X-Y triplet repeats where X is commonly proline and Y is commonly the hydroxylated form of proline-hydroxyproline (Linsenmayer, 1991; van-der Rest & Garrone, 1991; Burgeson & Nimni, 1992; Brown & Timpl, 1995). For a protein to be considered as a collagen, it must possess this basic structural unit somewhere within its structure and in terms of its function, it must be used structurally within the

ECM (Mayne, 1989).

Though apparently very specialised proteins, collagens are no different from any other protein in that they contain multiple domains (van-der Rest & Garrone, 1991), a combination of triple helices with globular and non-helical elements (Kuhn, 1987). In fact, although they are generally considered to be structural proteins, collagens are also involved in cell attachment and differentiation, can act as chemotactic agents, as antigens in immunopathological processes and can be the defective component in some pathological processes (Linsenmayer, 1991; Spotila *et al.,* 1994; Olsen, 1995). Collagens, then, appear to have a number of developmental and physiological functions.

Classifying the collagens into groups is extremely difficult. The fibrillar collagens, types I, II, III, V and XI, form lengthy uninterrupted collagenous domains (Mayne, 1989; Burgeson & Nimni, 1992) and are termed class I collagens (Burgeson & Nimni, 1992). Class 2 collagens include types IX and XII (Burgeson & Nimni, 1992) and possibly type XIV (van-der Rest & Garrone, 1991), and are also known as fibril associated collagens with interrupted triple helices or FACIT collagens. Class 3 collagens (Burgeson & Nimni, 1992) are a number of structurally and functionally distinct collagens, including type IV, type VI and type VII. Types VIII and X probably form their own group of non-fibril forming, short helix collagens as they are structurally similar, forming hexagonal lattices (Brown & Timpl, 1995). All non-fibril forming collagens may also be considered as a single large group (Brown & Timpl, 1995).

There are at least 5 genetically distinct collagen types found in articular cartilage (Eyre, 1991), types II, VI, IX, X and XI. Four of these, types II, IX, X, and XI are generally considered to be cartilage specific (Mayne, 1989; Trippel, 1990) although they have been found in other tissues (Eyre & Muir, 1976; Smith *et al.*, 1976; Timpl & Engle, 1987; Fitch *et al.*, 1989; Burgeson & Nimni, 1992; Ayad *et al.*, 1994). Small amounts of other collagens, including types XII and XIV are also present in cartilage (Ayad *et al.*, 1994).

Type II is the dominant collagen of cartilage (Kuhn, 1987; Mayne, 1989; Eyre, 1991), and is also found in the nucleus pulposus (Eyre & Muir, 1976; Burgeson & Nimni, 1992), the vitreous body of the eye (Smith *et al.*, 1976) and in several epithelially derived, embryonic chick matrices including the corneal epithelium, neuroepithelia and retinal pigmented epithelia (Fitch *et al.*, 1989; Kosher & Solursh, 1989). Type II collagen forms a network of fibrils in which cartilage proteoglycans are held (see below) (Mayne, 1989; Brown & Timpl, 1995). However, this collagen framework is in fact a hybrid polymer, as type IX and XI collagen are covalently bound via specific interactions to the type II fibrils (Mayne, 1989; Eyre, 1991).

Type II is a homotrimer of $\alpha$1(II) chains ($[\alpha 1(II)]_3$) (Kuhn, 1987; Trippel, 1990; Eyre, 1991; van-der Rest & Garrone, 1991) which are products of a single gene, COL2A1 (Eyre, 1991), 1487 amino acids in length and situated on chromosome 12 in humans (Ayad *et al.*, 1994). The chains form the classical triple helical structure (Trippel, 1990). $\alpha$1(II) has a higher content of

hydroxylysine and a higher glycosylation rate than the α1(I) chain of type I collagen (Kuhn, 1987).

Type IX collagen, a heterotrimer (α1(IX),α2(IX),α3(IX)) encoded by at least 2 different genes on separate chromosomes (1 and 6) (Ayad *et al.*, 1994), constitutes approximately 1% of the total collagenous material in articular cartilage (Trippel, 1990). It can be classed as both a collagen and a proteoglycan as it has a single CS chain covalently attached at a unique serine residue in one of its non-collagenous domains on the α2(IX) chain (Vaughan *et al.*, 1988; Mayne, 1989; Eyre, 1991; Burgeson & Nimni, 1992). The type IX molecules are covalently cross linked to type II fibrils (Wu & Eyre, 1989), and lie in a periodical manner along the surface of these fibrils, with its COL3 (Collagenous domain 3) and NC4 (Non-collagenous domain 4) domains projecting from the fibril surface (Vaughan *et al.*, 1988) which gives a bottle-brush like effect (Figure 1.4). The incorporation of type IX onto the surface of type II fibrils may control the lateral growth (Mayne, 1989; Trippel, 1990) or possibly the association (Eyre, 1991) of the type II fibrils. A further possible role for type IX collagen in articular cartilage is as an interface between the type II fibrils and the proteoglycan components of cartilage (Trippel, 1990; Eyre, 1991;Burgeson & Nimni, 1992). This hypothesis is taken one step further by Smith & Brandt (1992) who proposed that type IX is an intermediate molecule in the linking of type II fibrils. Due to its stereochemistry, it is unlikely that type IX can form a direct link between two type II fibrils. It therefore binds to an intermediary

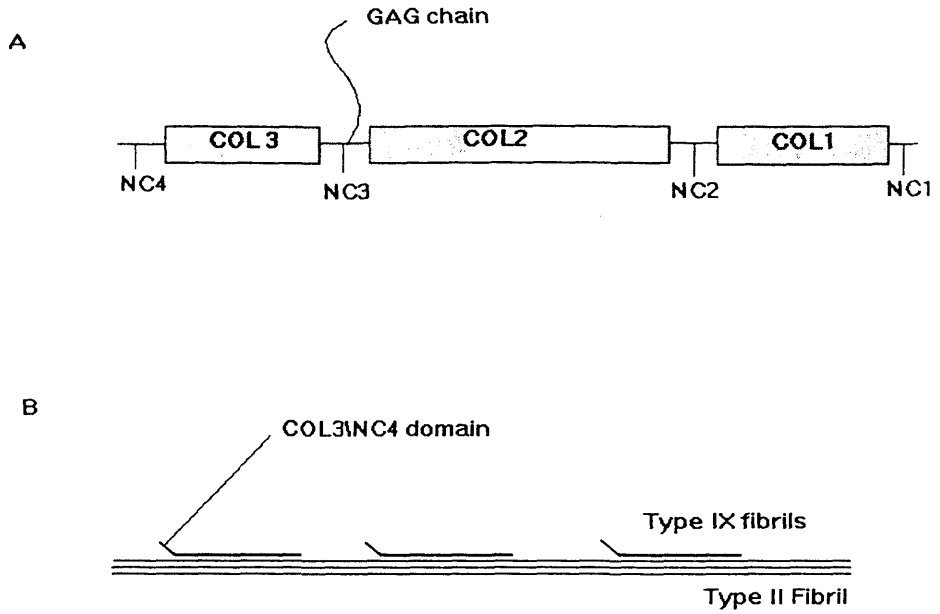



**Figure 1.4. A**. Diagrammatic representation of the structure of type IX collagen, COL are the collagenous (triple helical) domains. NC are the non-collagenous domain. The GAG chain is attached to the NC3 domain of α2(IX) (Vaughan *et al.*, 1988). **B**. Diagrammatic representation of the arrangement of type IX fibrils on the type II fibrils, showing the projection of the COL3/NC4 domains from the surface.

molecule most likely, a proteoglycan, which is linked to other type IX molecules attached to other type II fibrils.

Type XI collagen, originally known as 1α,2α,3α collagen (Burgeson & Hollister, 1979) and is therefore a heterotrimer (α1(XI),α2(XI),α3(XI)) of three distinct α chains, makes up between 5 and 10% of the total collagen in chick cartilage (Mayne, 1989), though in humans, it only makes up about 3% of the total collagen content (Trippel, 1990). It is present in the same fibrils as type II collagen (Mayne, 1989; Mendler *et al.*, 1989), though is found more concentrated in the fibril interior than on the surface (Mendler *et al.*, 1989; Trippel, 1990; Burgeson & Nimni, 1992). α1(XI) and α2(XI) are encoded by single genes on chromosomes 1 and 6 respectively, α3(XI) is identical to α1(II), but is more glycosylated (Ayad *et al.*, 1994). The role of type XI is not fully understood, but as with type IX, it is thought to be involved in the regulation of the size of type II fibrils, most likely in relation to their diameter (Lane & Weiss, 1975; Mayne, 1989; Ronziere *et al.*, 1990; Eyre, 1991).

Type X collagen, is a short helix homotrimer ($[\alpha1(X)]_3$), each chain being a total length of 680 amino acid residues, encoded on chromosome 6 in humans (Ayad *et al.*, 1994). It is thought to be produced solely by hypertrophic chondrocytes (Kielty *et al.*, 1985; Schmid & Linsenmayer, 1987; Kirsch & von der Mark, 1991; Reichenberger *et al.*, 1991), but has recently been shown to be deposited by pericytes (Schor *et al.*, 1995). It is believed to function during the vascularisation and calcification of cartilage in the endochondral ossification process during embryonic development and in the growth plate (Schmid &

Linsenmayer, 1987; Schmid *et al.*, 1990; Iyama *et al.*, 1991; Rooney & Kumar, 1993; Wallis, 1993) and perhaps has a mechanical function (Aspden, 1994; 1996). Structurally, it has been shown to assemble into a mat-like or hexagonal lattice structure when viewed under the electron microscope (Kwan *et al.*, 1991).

The final collagen component of articular cartilage is type VI collagen ($\alpha$1(VI), $\alpha$2(VI),$\alpha$3(VI)). This collagen has a triple helix of only 335-336 amino acids (van-der Rest & Garrone, 1991), which gives it a length of less than 300nm (Burgeson & Nimni, 1992). Structurally, it forms beaded filaments (van-der Rest & Garrone, 1991) which appear dumbbell shaped under the electron microscope (Burgeson & Nimni, 1992). It is widely distributed in varying amounts in most connective tissue (Timpl & Engle, 1987). In articular cartilage, it is concentrated in the pericellular domain around the chondrocytes (Poole *et al.*, 1988). As with types IX, X and XI, the function of type VI is not fully understood. It may be involved in the development and maintenance of the spatial separation of the distinct tissue components from the large banded fibrils within articular cartilage, or it may provide some form of structural unity to the tissue (Burgeson & Nimni, 1992). In a similar vein, it may act as an interface between the main collagen framework of the cartilage and the cartilage cells (van-der Rest & Garrone, 1991).

### 1.3.3 Proteoglycans:

Proteoglycans are an extremely diverse group of complex macromolecular glycoconjugates and are ubiquitous within the body. They are particularly concentrated in the ECM of connective tissues, especially in cartilage (Kuettner, 1992). The monomer molecule of a proteoglycan is assembled from a number of glycosaminoglycan chains attached to a central protein core, which under the microscope, gives the impression of a bottle brush (Trippel, 1990; Jeffery, 1994; Price, 1994).

In articular cartilage, proteoglycans are the second most prevalent organic component (Trippel, 1990) and provide the tissue with its ability to resist compressive loads and undergo reversible deformation. Much of the cartilage proteoglycan forms large aggregates by binding to hyaluronic acid via a small link protein (Trippel, 1990; Jeffery, 1994).

The proteoglycans in cartilage can form enormous aggregates (Mr 200,000 kD+). The main proteoglycan in articular cartilage is called aggrecan (Kuettner, 1992) because of its ability to form these aggregates. Approximately 90% of the aggrecan molecule is comprised of the GAG chains CS (a dimer of N-acetyl galactosamine with a sulphate ester on either the carbon 4 or carbon 6 atom and glucuronic acid) and KS (a sulphated dimer of N-acetyl glucosamine and galactose) (Kuettner, 1992). A region near the N-terminus contains most of the KS while a region of variable length at the C-terminus contains the CS (Heinegard & Oldberg, 1989; Korver *et al.*, 1990).

Two smaller proteoglycans containing the GAG dermatan sulphate (DS) are also present in articular cartilage (Korver *et al.*, 1990). These are biglycan, which contains 2 DS side chains, and decorin, which has only a single DS side chain (Kuettner, 1992). Another small proteoglycan like molecule called fibromodulin is also found in cartilage, lacking both CS and DS but contains KS (Wight *et al.*, 1991).

There is variation in the types and synthesis rate of proteoglycan and GAG chains between the defined layers of articular cartilage. For instance, there is an increase in GAG concentration with depth, especially in terms of KS content. Also, the size of the proteoglycan monomers decreases with depth. The superficial cartilage layer has been shown to have the lowest rate of proteoglycan synthesis (Korver *et al.*, 1990).

All of the components of the proteoglycan aggregates within articular cartilage are synthesised by the chondrocytes and are believed to largely undergo self-assembly (Jeffery, 1994).

### 1.3.4 Non-Collagenous Proteins:

Articular cartilage also contains a small percentage of proteins which are neither collagenous or proteoglycan in nature, these include anchorin, chondrocalcin, COMP, thrombospondin and fibronectin. These various molecules partake in interactions with other matrix molecules forming a network within which most of the ECM molecules are involved. Relatively little

work has been performed on these molecules (for review, see (Heinegard & Oldberg, 1989)). Chondrocytes are also able to produce a number of proteases (Martell-Pelletier *et al.*, 1991; McCollum *et al.*, 1991; Lotz *et al.*, 1995), growth factors and cytokines (Guerne *et al.*, 1994)

## 1.4. Cartilage Culture:

The formation of bone via endochondral ossification involves a series of complex maturational changes by the chondrocytes (Leboy *et al.*, 1989; Sullivan *et al.*, 1994). In mammals these cells undergo hypertrophy, ultimately producing a mineralised matrix, which forms the scaffolding for bone deposition (Sullivan *et al.*, 1994), however, in avians, there is a lack of mineralisation of the matrix (Roach & Shearer, 1989). This ordered progression through each of the steps, and the changes in gene expression that occur concomitant with these changes are crucial for normal skeletogenesis. Analysis of the mechanisms regulating both hypertrophy of the chondrocyte and mineralisation of the matrix are important therefore, in understanding the formation of bone, during development and repair of the skeleton and in pathological conditions such as osteoarthritis (OA). As with the majority of cell types, chondrocytes are open to investigation via *in-vitro* culture systems. However, chondrocytes are highly specialised cells, which *in-vivo* exist in a unique environment possibly without cell-cell contacts, surrounded entirely by ECM. This provides its own particular problems when maintaining

chondrocytes *in-vitro* for even short lengths of time, as chondrocytes require a rounded morphology to maintain the chondrocyte phenotype (Archer *et al.*, 1982; Benya & Shaffer, 1982; Archer *et al.*, 1990, Bonaventure *et al.*, 1994)

Culturing both pre-chondrogenic mesenchyme and chondrocytes has allowed us an insight into many of the developmental and metabolic processes of these highly specialised cells.

### 1.4.1. Culture Models:

Much of the work involving the study of chondrogenesis used *in-vitro* models (Paulson *et al.*, 1994; see Archer *et al.*, 1983 & Archer *et al.*, 1984 for reviews), as chondrogenesis *in-vitro* can recapitulate the steps seen *in-vivo* (Paulson *et al.*, 1994). A commonly used model is the limb bud mesenchyme from the developing chick embryo. Tissue isolates taken from the early limb bud will undergo chondrogenesis after cell dissociation and plating (Caplan & Pechak, 1987; Solursh, 1989). However, culture on plastic requires cells to be plated at greater than confluent densities for chondrogenesis to occur (Umansky, 1966; Ahrens *et al.*, 1977). Single mesenchymal cells can also become chondrogenic if the cell is maintained as a sphere by culture as a cell suspension (Solursh & Reiter, 1975), maintained in a rounded configuration on tissue culture plastic (Archer *et al.*, 1982), or within a gel, including agarose or hydrated collagen gels (Solursh *et al.*, 1982).

Chondrocytes from a variety of species, including rodent (Closs *et al.*,

1990; Zimmerman *et al.*, 1994), chick (Farquharson & Whitehead, 1994; Sullivan *et al.*, 1994; Iwamoto *et al.*, 1995), bovine (Hinek *et al.*, 1987; Alini *et al.*, 1996) and human (Bassleer *et al.*, 1992; Shingu *et al.*, 1993; Guerne *et al.*, 1994; Gunther *et al.*, 1994; Iwamoto *et al.*, 1994) have all been used in culture to study various aspects of chondrocyte metabolism. However chondrocytes grown as monolayers are unable to imitate the spatial relationships that occur between the cells and matrix *in-vivo* (Farqhuarson & Whitehead, 1994). When cultured *in-vitro,* at low densities, the chondrocyte phenotype is unstable and is susceptible to modulation (Kosher, 1983). They are unable to maintain their rounded shape and have a tendency to dedifferentiate and become fibroblastic in nature with a concomitant change in collagen expression from type II to type I (Mayne *et al.*, 1976; Kirsch *et al.*, 1992; Bonaventure *et al.*, 1994).

The chondrocyte phenotype is related to it being maintained in a rounded configuration (Archer *et al.*, 1990). Conditions which promote cell flattening, *in-vitro* at least, inhibit chondrogenic expression (Solursh, 1989). To achieve a rounded morphology *in-vitro*, it is necessary to culture cells under conditions which prevent cell flattening. Preventing cell surface adhesion is one method of doing this, and several techniques have been used successfully to do this. These include culture over (Castagnola *et al.*, 1988; Archer *et al.*, 1990; Kirsch *et al.*, 1992) or in (Aulthouse, 1994; Gunther *et al.*, 1994) agarose, culture in collagen gel (Gibson *et al.*, 1982), methyl cellulose (Horton & Hassel, 1986), alginate (Grandolfo *et al.*, 1993; Bonaventure *et al.*, 1994; Hauselmann *et al.*, 1994) or on bacteriologic dishes (Pacifici *et al.*, 1991). All of these stabilise the

chondrocytic phenotype and can promote cell hypertrophy (Kirsch *et al.*, 1992).

A further method used to stabilise the chondrocytic phenotype in culture involves plating the cells at high densities by centrifugation into a pellet within an eppendorf (Farquharson & Whitehead, 1994) or by culture as 'micromasses', whereby large numbers of cells are plated in a small droplet of growth medium (Ahrens *et al.*, 1977; Langille & Solursh, 1990). This aids in maintaining a rounded shape and expression of differentiated characteristics typical of chondrocytes e.g. type II and X collagen (Farquharson & Whitehead, 1994). Finally, the culture of cartilage explants and developing cartilage rudiments *in-vitro* by a number of workers, has allowed the study of chondrocytes in their native environment, e.g. (Closs *et al.*, 1990; Kistler *et al.*, 1990; Martel-Pelletier *et al.*, 1991; Cole *et al.*, 1992; Curtis *et al.*, 1992; Lafeber *et al.*, 1993; Rayan & Hardingham, 1994; Posever *et al.*, 1995; Roach *et al.*, 1995).

Although the physical culture conditions influence the phenotype of a cultured chondrocyte, chemical conditions are just as vital. Alteration of the chemical environment allows manipulation of virtually all aspects of cell metabolism.

### 1.4.2 Chondrocyte Metabolism in Culture:

Hypertophy is considered by many to be the final and terminal stage in chondrocyte maturation before they degenerate and die (Galotto *et al.*, 1994; see Roach *et al.*, 1995). Chondrocyte hypertrophy can be enhanced in culture by

altering the environmental conditions (Ekanayake & Hall, 1994), allowing the study of the cellular, biochemical and molecular changes associated with this vital phenotypic change. This should help further develop the understanding of bone formation during development and skeletal repair (Sullivan *et al.*, 1994) and possibly during pathological conditions.

Two common inducers of chondrocyte hypertrophy are ascorbate (Leboy *et al.*, 1989; Ekanayake & Hall, 1994; Sullivan *et al.*, 1994) and retinoic acid (Iwamoto *et al.*, 1994; Sullivan *et al.*, 1994). A number of other compounds have also been shown to induce chondrocyte hypertrophy. These include calcium-β-glycerophosphate (Thomas *et al.*, 1990), elevated calcium levels (Bonen & Schmidt, 1991) and addition of thyroxin + insulin or insulin like growth factor-1 (IGF-1) (Sullivan *et al.*, 1994). The hypertrophic phenotype can be identified by a number of markers, including an increase in alkaline phosphatase (ALP) activity (Iwamoto *et al.*, 1994; Sullivan *et al.*, 1994), production of type I collagen, osteonectin and bone gla protein (Closs *et al.*, 1990) and the appearance of matrix vesicles (Sullivan *et al.*, 1994). However, the only specific marker for chondrocyte hypertrophy is the production of type X collagen (Schmid *et al.*, 1991; Sullivan *et al.*, 1994). *In vivo*, the hypertrophic state is the preceding step to mineralisation and bone formation during development (Galotto *et al.*, 1994; Sullivan *et al.*, 1994). Chondrocyte cultures can be induced to mineralise by the addition of vitamin D (Hinek *et al.*, 1987) and β-glycerophosphate (Ekanayake & Hall, 1994). The mineralisation of cartilage is closely associated with an increase in ALP activity, as is seen in all

mineralising tissue (Sullivan *et al.*, 1994), an increase in deposition of the C-propeptide of type II collagen (chondrocalcin) (Hinek *et al.*, 1987) and type X collagen (Reginato *et al.*, 1993).

### 1.4.3 Cytokines and Growth Factors:

*In-vivo,* chondrocytes are subject to modulation by both cytokines and growth factors, which are responsible for control of all aspects of cell metabolism. However, in culture, chondrocytes are removed from these influences and as such, the effects of single, or combinations of specific factors can be identified and monitored.

In the broadest aspect, chondrocytes can be classed as having two distinct functional programs, an anabolic program whereby there is synthesis of ECM macromolecules and protease inhibitors, and cells are seen to proliferate, and a catabolic program involving suppression of ECM synthesis, secretion of proteases and inhibition of cellular proliferation (Lotz *et al.*, 1995). A number of studies have been performed on the effects of cytokines and growth factors on chondrocyte functions (McCollum *et al.*, 1991; Pujol *et al.*, 1991; Shingu *et al.*, 1993; Guerne *et al.*, 1994; Gunther *et al.*, 1994; Iwamoto *et al.*, 1995). A number of the cytokines and growth factors that influence chondrocyte metabolism are in fact products of the cells themselves. Chondrocytes can produce interleukin-1 (IL-1), transforming growth factor β (TGF-β), platelet derived growth factor AA (PDGF-AA), basic fibroblast growth factor (bFGF)

and IGF-1 (Guerne *et al.*, 1994). It appears likely that chondrocytes may play a key role in regulating their own development, maturation, catabolic and anabolic processes.

An important cytokine in chondrocyte catabolism is IL-1. It induces catabolic responses in chondrocytes by stimulating protease expression (Martel-Pelletier *et al.*, 1991; McCollum *et al.*, 1991; Lotz *et al.*, 1995) and suppressing α1(II) procollagen mRNA expression (Goldring *et al.*, 1994). Suprisingly, IL-1 has been shown to increase the production of tissue inhibitors of metalloproteases (TIMP) (Shingu *et al.*, 1993), though this was only shown to happen at high doses of IL-1 (Martel-Pelletier *et al.*, 1991). However Shingu *et al.*, (1993) showed an overall increased ratio of collagenase to TIMP in the chondrocyte supernatant when IL-1 induces TIMP production.

IL-1 therefore, in particular, plays a vital role in the balance between TIMP's and matrix metalloproteases (MMP) and is likely to be involved in the degradation of the cartilage matrix. Tumour necrosis factor-α (TNF-α) also increases the MMP/TIMP ratio (Shingu *et al.*, 1993) and is likely also to be involved in the catabolism of cartilage. TNF-α has also been shown to down regulate the IL-1 receptor present on the chondrocyte surface (McCollum *et al.*, 1991) and as such may also be involved in the regulation of the effects of IL-1.

TGF-β1 has been shown to suppress IL-1 induced destruction of articular cartilage both *in-vivo* and *in-vitro* (van Beuningen *et al.*, 1993), though only if added after addition of the IL-1 (Pujol *et al.*, 1991). Functionally, TGF-β1 partly controls the synthesis of ECM components and reduces the synthesis and

secretion of the MMP while stimulating production of TIMP (Gunther *et al.*, 1994). The effects are therefore antagonistic to those caused by IL-1, and it seems probable that a balance between these two factors plays a role in balancing anabolism and catabolism of cartilage. TGF-β has also been shown to be mitogenic to human chondrocytes, increasing the rate of proliferation in culture, as have bFGF, PDGF-AA and IGF-1, though all to a lesser extent (Guerne *et al.*, 1994).

A further factor shown to have an influential effect on chondrocyte metabolism is parathyroid hormone (PTH). Along with bFGF, PTH has been shown to be involved in the maturation process of chondrocytes (Iwamoto *et al.*, 1995). The authors also noted that the effects of these factors on the cells were dependent on the stage of development of the cells before treatment. The more mature the cells were, the less sensitive they became to the factors.

### 1.4.4 The Fate of the Chondrocyte:

Little information is available on the eventual fate of chondrocytes. The general belief is that hypertrophic chondrocytes are terminally differentiated cells (Pacifici *et al.*, 1990), which degenerate and die (see Roach *et al.*, (1995)), though an alternative view (see Roach *et al.*, (1995)), and evidence, suggests that some hypertrophic chondrocytes can divide asymmetrically, one cell remaining viable and producing cells of an osteogenic phenotype, the other destined to die by apoptosis (Descalzi Cancedda *et al.*, 1992; Roach *et al.*,

1995). It has been proposed that chondrocytes expire by apoptosis (Hatori *et al.*, 1995).

In culture, chondrocytes have been shown to survive for weeks at high cell densities in the absence of serum, exogenous proteins, signalling molecules or other cell types. In contrast, under these conditions at low densities, the cells tend to die, showing the morphological features of apoptosis (Ishizaki *et al.*, 1994). Chondrocytes in culture can also be induced to undergo apoptosis via the addition of extrinsic molecules. Nitric oxide (NO) from nitroprusside induces apoptosis in cultured chondrocytes, though IL-1 stimulation of NO did not cause apoptosis (Blanco *et al.*, 1995). The authors also showed that under certain conditions, the cells underwent necrosis.

It appears then that cultured chondrocytes can undergo both apoptosis and necrosis, allowing the study of the reasons how and why these processes occur. The underlying factors leading to either of these fates is yet to be determined, though the fact that most cartilage is replaced during both development and growth of bone shows that one of its roles is to provide a substratum for the laying down of mineralised tissue. This is a very ordered and timely process, and therefore the necessity for the cartilage to 'disappear' at these stages is a reason for its programmed removal and does provide a possibility, at least, as to why chondrocytes should undergo apoptosis.

## 1.5. Cartilage Pathology:

Despite its uniqueness as a tissue, cartilage pathology is invariably linked to the pathology of the entire joint (Hulth, 1990). The general response of cartilage to joint pathology is a breakdown of the ECM and ultimate destruction of the cartilage (Figure 1.5)

### 1.5.1 Cartilage Homeostasis:

Normal adult cartilage is maintained by a balance between new matrix synthesis and matrix degradation, the rates of each being equal, resulting in no net loss of tissue (Pelletier *et al.*, 1993). This consistency is dictated in part by an equilibrium between enzymes responsible for catabolising cartilage matrix and inhibitors of these enzymes. Thus chondrocyte activity is responsible for maintaining articular cartilage homeostasis (Heinegard & Saxne, 1991; Testa *et al.*, 1994). Chondrocyte metabolic activity and thus cartilage homeostasis, is regulated by growth factors and cytokines (Pelletier *et al.*, 1993). These agents are associated with both anabolism and catabolism of cartilage tissue e.g. growth factors, such as TGF-β, are involved in the formation of connective tissue, whereas cytokines, such as IL-1 and TNF-α, are associated with the degradation of the tissue (Chandrasekhar *et al.*, 1993; Pelletier *et al.*, 1993).

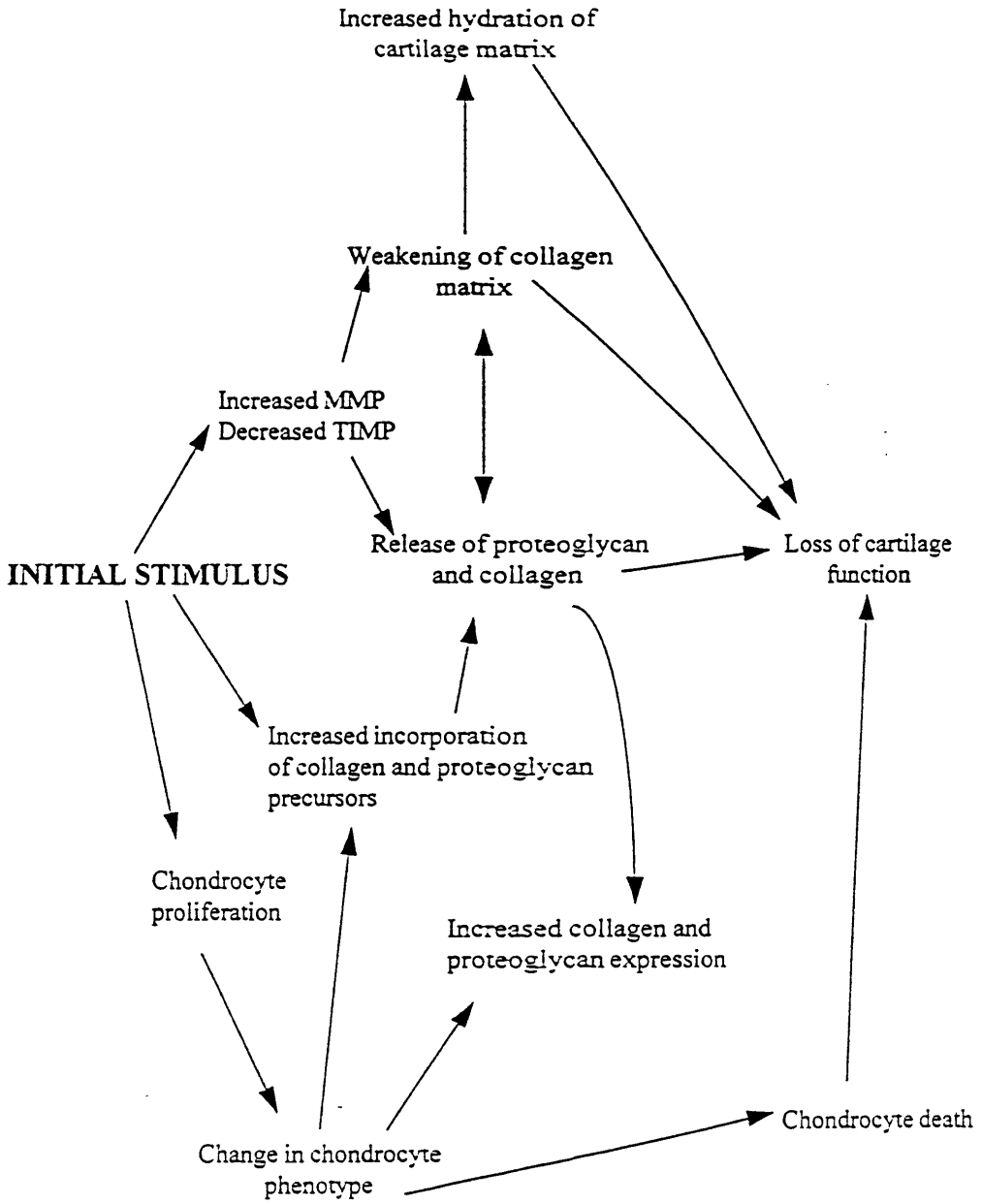



**Figure 1.5.** Simplified flow diagram of the degeneration of articular cartilage irrespective of the cause.

### 1.5.2 Joint Disease and Cartilage Degeneration:

Joint disease is not a single entity and is generally only a part of a wider disorder of the musculoskeletal system (Bullough, 1997).

A common feature of most joint disease is the degeneration of the articular cartilage. However, this is more likely to be a symptom of the disorder as opposed to the cause of the disease. Regardless of the cause, the response of the articular cartilage (and the other tissues of the joint) is characterised by a set of basic (programmed) cellular and tissue responses (Bullough, 1997). There is a uniformity of the type of response of the cartilage, regardless of the initiating factor (Peltonen, 1990). In joint disease, there is a loss of the balance of ECM macromolecule synthesis and degradation, the macromolecules of the cartilage ECM become degraded by proteolytic enzymes, causing destruction of the ECM integrity and a change in the properties of the tissue (Heinegard & Saxne, 1991).

### 1.5.3. Osteoarthritis:

Osteoarthritis (OA) is a disease of complex aetiology (Dean, 1991), often considered to be a slowly progressive disorder occurring late in life (Mankin, 1974b). In fact, it cannot be classed as a single disease entity, but instead is a common end-point of cartilage and joint pathology (Meachim, 1972; Lohmander, 1994). There are a number of clinical variants proposed as predisposing conditions for OA, including erosive OA, diffuse idiopathic skeletal hyperostosis and chondromalacia patellae (Hamerman, 1989). OA can

affect axial joints such as those between the vertebrae of the spine, though more commonly, it affects the peripheral joints such as the hip and knee (Howell *et al.*, 1976; Hamerman, 1989). The result of OA is a loss of the normal function of the joint due to a breakdown of the articular cartilage (Dean, 1991). Other structures within the joint are also adversely affected by the disease, including the subchondral bone, the ligaments and the synovium (Dean, 1991; Grynpas *et al.*, 1991). In fact, changes in these extracartilaginous structures have been considered to be the primary initiating factor in the development of OA (Dean, 1991)

The cause of OA is unknown, however, there are two major classes of the disease seen in patients. Primary, or idiopathic, OA where the aetiology is unclear, and secondary OA, where there is a pre-disposing condition (Howell *et al.*, 1976; Hamerman, 1989).

At the macroscopic level, OA appears as a focal disease (Mankin *et al.*, 1971; Mankin, 1974b; Howell *et al.*, 1976). The normal, glistening appearance of hyaline cartilage is replaced by yellow/brown spots (Howell *et al.*, 1976). In its early stages there is limited cartilage degradation (Poole *et al.*, 1993). The cartilage becomes fibrillated or frayed with penetrating fissures or clefts and some loss of cartilage tissue (Meachim, 1972; Hamerman, 1989; Nerlich *et al.*, 1993; Poole *et al.*, 1993). As the disease becomes more severe, the fissures and fibrillation become more prominent (Hwang *et al.*, 1992) and cartilage erosion becomes severe, often with eburnation, and focal exposure of the subchondral bone (Meachim, 1972; Howell *et al.*, 1976; Nerlich *et al.*, 1993). Even in early

OA, there are also changes occurring in the underlying subchondral bone (Grynpas *et al.*, 1991). There is a remodelling of the bone structure which becomes more prominent in the later stages (Jeffery, 1973), the thickness of the subchondral bone increases significantly, and there is a loss in density of the tissue shown by an abnormally low pattern of mineralisation compared to age matched controls (Grynpas *et al.*, 1991). There is bony sclerosis, cyst formation and the formation of osteophytes at the joint margin, representing renewed growth of cartilage and bone (Mankin, 1974b; Hamerman, 1989; Hulth, 1993; Archer, 1994). The tidemark between the bone and cartilage becomes violated by blood vessels (Mankin, 1974b).

### 1.5.4 Osteoarthritis and Collagen:

Chondrocytes in normal adult articular cartilage are biosynthetically similar to foetal chondrocytes in that they express collagen types II, VI, IX and XI (von der Mark *et al.*, 1992). A number of studies have been performed to investigate whether there is a change in the phenotype of chondrocytes and/or a change in the ECM of articular cartilage during OA (Adam & Deyl, 1983; Hoyland *et al.*, 1991; Aigner *et al.*, 1992; von der Mark *et al.*, 1992; Aigner *et al.*, 1993a & b; Nerlich *et al.*, 1993; Hollander *et al.*, 1994). Maroudas *et al.*, (1985) showed an increase in the hydration of articular cartilage during early OA, which was suggested to be due to a weakening of the collagen network. This process is thought to be an important early event in the process of the disease (Hamerman,

1989). Degradation and loss of collagen from cartilage matrix could contribute to the degeneration of cartilage and loss of its function in arthritic patients (Hollander *et al.*, 1994), and a decrease in collagen content has been demonstrated in OA cartilage (Venn & Maroudas, 1977).

Normal cartilage shows only a very low level of collagen synthesis (Repo & Mitchell, 1971). However, articular chondrocytes are able to respond to degeneration by increasing their production of type II collagen (and proteoglycan) up to a certain degree of tissue damage (Lipiello *et al.*, 1977; Ryu *et al.*, 1984), demonstrated by an increase in $^{3}$H-proline incorporation by the chondrocytes and measurement of the specific activity of hydroxyproline (Lipiello *et al.*, 1977). There is evidence to suggest that significant alterations in the collagen expression of chondrocytes do occur during OA (Adam & Deyl, 1983; von der Mark *et al.*, 1992; Aigner *et al.*, 1993a & b), particularly for a phenotypic switch of the chondrocytes to produce collagen types I, III (Nimni & Deshmukh, 1973; Adam & Deyl, 1983; Aigner *et al.*, 1993a; Nerlich *et al.*, 1993) and X (Hoyland *et al.*, 1991; von der Mark *et al.*, 1992; Aigner *et al.*, 1993b).

Type I and III collagen have been identified immunohistochemically in OA articular cartilage in the pericellular matrix of some chondrocytes (Adam & Deyl, 1983; Nerlich *et al.*, 1993), and the mRNA for type III collagen, but not type I collagen, has also been found in OA cartilage (Aigner *et al.*, 1993a). Biochemical analysis has demonstrated that most of the collagen in OA cartilage is still type II (Ronziere *et al.*, 1990). Although levels of types I and III

collagen in OA cartilage may not be significant relative to the amount of type II collagen (Nerlich *et al.*, 1993), the minor differences are indicative of important modifications in chondrocyte metabolism and the collagen network during cartilage degeneration in OA (Ronziere *et al.*, 1990).

Type X collagen is believed to function during the calcification of cartilage during endochondral ossification (Schmid & Linsenmayer, 1987). Cartilage mineralisation and the formation of bone on calcified cartilage are thought to be key phases during bone remodelling in OA (Skoloff, 1987). The presence of type X collagen has been shown in normal articular cartilage immunologically at the cartilage surface (von der Mark *et al.*, 1992) and at the mRNA level in some chondrocytes adjacent to the calcified cartilage layer (Hoyland *et al.*, 1991). In OA cartilage, type X collagen has been shown immunohistochemically around chondrocyte clusters in fibrillated, severely damaged OA cartilage in the upper and middle zones, in hypertrophic cells (von der Mark *et al.*, 1992; Aigner *et al.*, 1993b) and within the chondrocytes of the calcified layer (Hoyland *et al.*, 1991). The staining appears usually as discrete regions both intra- and pericellularly (von der Mark *et al.*, 1992). However, mRNA transcripts for type X collagen could not be found in areas of positive immunohistochemical staining, suggested to be due most likely to low levels of transcript (Aigner *et al.*, 1993b).

The expression and distribution of type II collagen has also been studied extensively (Aigner *et al.*, 1992; von der Mark *et al.*, 1992; Aigner *et al.*, 1993a; Hollander *et al.*, 1994). Hollander *et al.*, (1994) showed, in OA cartilage,

increased staining for denatured type II collagen, using an antibody specific to the denatured form. This was interpreted as being an increase in denaturation and a decrease in the total amount of type II collagen within the matrix. The damage was most evident in the superficial and upper mid zones, which coincides with the initial loss of proteoglycan from the matrix. Von der Mark *et al.*, (1992) showed two patterns of staining for type II collagen, usually, both patterns being present in the same sample. The first pattern was distinguished by an almost complete lack of staining in the upper zone and in the inter-territorial matrix of the middle zone. However, strong staining was seen in the pericellular matrix of the middle zone chondrocytes. This strong staining was attributed to the synthesis of new collagen. The second pattern showed a loss of staining within the pericellular matrix resulting in a halo type effect, hypothesised to be due to the action of proteases (von der Mark *et al.*, 1992).

Finally, among the minor collagens, no intact type IX collagen has been extracted from human OA cartilage (Brierley *et al.*, 1991), though fragments of this collagen have been isolated (Ronziere *et al.*, 1990). Type VI collagen appears enriched within the pericellular domain where it is normally localised (Ronziere *et al.*, 1990; Brierley *et al.*, 1991) and little is known about type XI.

It appears that the collagenous network of articular cartilage is likely to undergo alteration during the disease process of OA. The reasons for the changes in expression and distribution of the original cartilage collagen types, and the newly expressed collagens, however, are yet to be fully elucidated.

### 1.5.5 Osteoarthritis and Proteoglycans:

The earliest and most prominent change in OA cartilage, at the histological level is a progressive increase in the loss of proteoglycans (Ryu, 1984; Lafeber *et al.*, 1992a & b; 1993), shown by a moderate decrease in metachromatic staining (Mankin, 1974b). The release of proteoglycan from OA cartilage is believed to be greater than that from normal cartilage (Yamada *et al.*, 1987). As the disease progresses, there is a progressive loss of colour with the metachromatic stains, such as alcian blue, and the orthochromatic stains, such as safranin-O (Mankin, 1974b), which can be explained by the extensive removal of aggrecan seen in severe OA (Poole *et al.*, 1993). The levels of proteoglycan within normal matrix depends upon a balance between their rate of synthesis and the rate at which they become lost from the matrix (Grushko *et al.*, 1989). During OA, there is evidence for an increase in the release of proteoglycan as increased levels of aggrecan have been described in the joint fluid of OA (Lohmander, 1994).

Despite the loss of proteoglycans (Ryu, 1984; Lafeber *et al.*, 1992a & b; 1993), there is also evidence for increased metabolic activity of OA chondrocytes, originally shown by Collins & McElligott (1960). This hypermetabolism of chondrocytes is proportional to increasing severity of the disease in mild to moderate OA. However, as the disease progresses in its severity, the metabolism of the cells decreases markedly, suggesting that the capacity of the cell to respond to the damage becomes exceeded (Mankin *et al.*, 1971; Mankin, 1974b). This point has been identified on a histological-

histochemical scale devised by Mankin *et al.*. (1971), based on the loss of matrix staining with haemotoxylin & eosin and safranin-O-fast green. The greater the loss of staining, the higher the grade. The failure of the cells occurs at grade 10 on this scale (Mankin *et al.*, 1971). The tissue is also enzymatically degraded more rapidly in comparison to normal cartilage tissue (Mankin *et al.*, 1981) which presumably accounts for the net loss of proteoglycan.

The synthesis of proteoglycans in OA cartilage has been extensively studied (Mankin *et al.*, 1971, 1981; Christensen & Reiman, 1980; Ryu *et al.*, 1984; Grushko *et al.*, 1989; Lafeber *et al.*, 1993). New proteoglycan synthesis has been shown by the incorporation of radioactive sulphate $^{35}SO_4$ (Mankin *et al.*, 1971; 1981; Ryu *et al.*, 1984; Grushko *et al.*, 1989; Lafeber *et al.*, 1992b, 1993), $^{3}$H-glucosamine (Mankin *et al.*, 1981; Ryu *et al.*, 1984) and $^{3}$H-glycine (Ryu *et al.*, 1984). The general and now widely accepted view is for an increased incorporation of radiolabelled substrate of both sulphate (up to 13 fold greater than normal (Ryu *et al.*, 1984)) and amino sugar. Specifically, hyaluronan has been shown to be synthesised in excess of the proteoglycan subunit in OA cartilage. Much of this hyaluronan subsequently fails to aggregate. There has also been shown to be a decrease in glucosamine but no change in galactosamine, associated with a decrease in KS but no change in CS (Mankin *et al.*, 1981; Ryu *et al.*, 1984). Selective histochemical staining of GAG also shows a change in the distribution of these entities in OA cartilage (Christensen & Reiman, 1980). These results however are disputed by the work done by Grushko *et al.*, (1989) who showed via a topographical study

comparing GAG and proteoglycan synthesis in cartilage from OA joints and from cartilage from the same region in a normal joint, that there is no overall increase in the rate of proteoglycan production in OA cartilage compared to normal.

*In-vitro* work has shown that freshly isolated OA tissue shows an enhanced rate of proteoglycan synthesis which decreases below that of normal cartilage after several days in culture (Lafeber *et al.*, 1992a, 1992b; Venn *et al.*, 1995). This suggests that the hypermetabolism of OA chondrocytes is in fact a reversible process and is not a permanent change in phenotype (Venn *et al.*, 1995).

There is, therefore, evidence to suggest that during OA, the chondrocytes within the articular cartilage respond to the disease by mounting a biosynthetic response, which is considered to be an attempt at repair (Mankin, 1974b; Mankin *et al.*, 1981; Ryu *et al.*, 1984) By the use of radiolabelled precursors of both collagen and proteoglycan, there is evidence for the attempted repair of OA damaged cartilage by a new wave of synthesis, though this overexpression may not be a permanent feature of the cells. However, the quality and quantity of these new products still require further evaluation (Ryu *et al.*, 1984).

It should further be noted that deep lacerative injury to the articular cartilage, which violates the underlying bone also leads to a repair response from the cartilage and bone marrow. This response, however, is different from that occurring during OA as it is mediated via the vasculature with the

formation of a fibrin clot which subsequently forms fibrocartilage. There is a brief burst of synthetic activity by chondrocytes at the edges of the newly formed mass, but there is no extended period of cartilage biosynthesis (for review, see (Mankin *et al.*, 1974a)).

### 1.5.6 Osteoarthritis and the Chondrocyte:

The fate of chondrocytes during the degenerative processes of OA has received little attention in comparison to the collagenous and proteoglycan components of cartilage. During the disease there is a noticeable proliferation of chondrocytes inside their lacunae (Mankin, 1974b; Howell *et al.*, 1976), forming clusters of new cells called chondrones (Archer, 1994). This re-initiation of mitosis has been studied with the use of $^{3}$H-thymidine (Mankin *et al.*, 1971). The study showed that incorporation rates of $^{3}$H-thymidine and therefore rate of DNA synthesis and mitotic activity increased proportionally with the severity of the disease, up to the same point where $^{35}SO_4$ incorporation falls off, then the rate decreases. However, the study also showed that the levels of DNA remained essentially constant, the conclusion drawn was that chondrocyte proliferation was matched by cell death (Mankin *et al.*, 1971).

The ultimate fate of articular chondrocytes during OA is unknown. It is generally accepted that articular chondrocytes persist throughout life (though the question posed earlier remains!!!) with very little turnover due to a limited or even possibly non-existent mitotic ability *in-situ* (Mankin, 1974b). It would

appear most likely, that as the cartilage is eventually completely eroded, the chondrocytes must die. During endochondral ossification, hypertrophic chondrocytes and their matrix are resorbed and ultimately replaced by bone (Galotto *et al.*, 1994; Gibson *et al.*, 1995a). The fate of the chondrocyte during the process of endochondral ossification is not fully known. The mechanisms by which the death of the chondrocytes can occur are open to question. There are two known mechanisms of cell death, necrosis and apoptosis (Cohen, 1996). During endochondral ossification, it is generally believed that chondrocytes degenerate and die (see Roach *et al.*, (1995)) presumably by necrosis, though it has been proposed that chondrocytes can undergo apoptosis (Hatori *et al.*, 1995; Roach *et al.*, 1995). Also, in developing chick cartilage, though there is a distinct lack of endochondral ossification (Fell, 1925; Roach & Shearer, 1989), some chondrocytes have been shown to undergo apoptosis along the rim of the invasive vasculature (Gibson *et al.*, 1995a). The question remains, do articular chondrocytes, which appear to become hypermetabolic up to a certain point in the disease before their metabolism decreases, then slowly degenerate and die by necrosis, or is there some form of programmed cell death, which is triggered once the disease reaches a certain state?

### 1.5.7 The Degradation of Cartilage During Osteoarthritis:

Articular cartilage degeneration involves the degradation of both the collagen and proteoglycan components of the tissue and the cells. It is generated by

proteolytic enzymes (Dean *et al.*, 1991; Pelletier *et al.*, 1993; Testa *et al.*, 1994). A number of proteases are involved in the pathology of cartilage, the major enzymes being members of the matrix metalloproteinase (MMP) family (Dean, 1991; Martell-Pelletier *et al.*, 1991; Pelletier *et al.*, 1993; Poole *et al.*, 1993). There are now 13 known MMPs, the thirteenth, MMP-13 or collagenase-3 was first identified in breast carcinomas (Freije *et al.*, 1994) and has subsequently been shown to be expressed at very low levels in normal cartilage and at higher levels in OA cartilage (Reboul *et al.*, 1996). The MMPs range in size from 28kD to 92kD (Dean, 1991), all of which have a number of common properties. They all degrade at least one component of the ECM, all contain a zinc ion and therefore can be inhibited by chelating agents such as EDTA, all are secreted in a latent form, which requires activation by proteolytic activity, and all are inhibited by specific inhibitors- tissue inhibitors of metalloproteinase (TIMP) (see (Matrisian, 1990) for concise review). A number of other proteases which do not require a metal ion for activity are also involved in cartilage matrix degradation, including serine and cysteine proteases (Dean, 1991).

MMPs constitute a single evolutionary protein superfamily based on their structure and function, but can be separated into 3 subclasses- the collagenases, the gelatinases (type IV collagenases) and the stromelysins (Matrisian, 1990; Reboul, 1996). Of these three subclasses, the collagenases and the stromelysins are particularly important in cartilage degradation (Mort *et al.*, 1993; Pelletier *et al.*, 1993; Shingu *et al.*, 1993; Testa *et al.*, 1994).

All MMPs form a high affinity, 1:1 molar ratio complex with TIMPs, in

a non-covalent manner (Matrisian, 1990; Shingu *et al.*, 1993). TIMPs are produced by the same cells that are responsible for producing the MMPs (Shingu *et al.*, 1993). TIMP is a protein of approximately 28.5kD (Dean, 1991), and at least two, TIMP-1 and TIMP-2, are known to exist in humans (Pelletier *et al.*, 1993).

Evidence suggests (Dean *et al.*, 1989, Pelletier *et al.*, 1990), and it is now widely accepted, that OA lesions result from an imbalance between the activities of MMPs and their inhibitors within the cartilage. In early OA joints, there is an increase in anabolism to counteract the increased catabolic changes in the tissue (Lohmander, 1994; Testa *et al.*, 1994). However, as discussed, there is a point where the chondrocytes become unable to match the rate of catabolism, resulting in net loss of matrix (Pelletier *et al.*, 1993). It is still not fully known whether this loss is due to an increase in MMP activity or a decrease in TIMP activity, or both. In normal cartilage, TIMP is in slight excess over MMP content (Woessner Jr. & Gunja Smith, 1991). The levels of MMPs have been shown to be increased in OA cartilage compared to those in the normal tissue (Pelletier *et al.*, 1983; Testa *et al.*, 1994; Reboul *et al.*, 1996) and enzymatic degradation of the ECM is more rapid. The levels of collagenase and stromelysin correlate with the severity of the disease (Pelletier *et al.*, 1993). It is currently thought that TIMP levels do not increase to match this increase in MMP levels (Dean *et al.*, 1989; Pelletier *et al.*, 1990). Interstitial collagenase (MMP-1) is responsible for the degradation of collagens I, II and III by cleavage

at a single, sequence specific site in the triple helix, causing unwinding of the collagen fibre to give two fragments in a ratio of 3:1 of the original length (Matrisian, 1990; Dean, 1991; Mort *et al.*, 1993; Reboul *et al.*, 1996). This results in a denatured gelatin, which is then susceptible to further cleavage by other non-MMPs, such as cathepsins (Matrisian, 1990; Dean, 1991). However, MMP-1 has little effect on the other matrix components (Shingu *et al.*, 1993). Stromelysin (MMP-3) degrades proteoglycan, laminin, fibronectin, link protein and type II collagen in the non-helical N-telopeptide region (Mort *et al.*, 1993; Pelletier *et al.*, 1993; Shingu *et al.*, 1993). However, the complete degradation of cartilage proteoglycan is rather more complex than this, and requires a number of enzymes in addition to stromelysin. Sulphatases, glycosidases and non-MMPs are also required (Dean, 1991). The proteolytic degradation of aggrecan is also typical of arthritis pathology (Fosang *et al.*, 1996). Fragments of aggrecan distinct from those generated by MMP's have been isolated from human OA cartilage (Lark *et al.*, 1997). A putative enzyme termed aggrecanase is predicted to be responsible for this non-MMP mediated catabolism (Ilic *et al.*, 1995; Lohmander *et al.*, 1993). The combined effect of these enzymes is to reduce the proteoglycan to a mixture of peptidoglycans (Testa *et al.*, 1994).

The most important source of hydrolytic enzymes in OA is thought to be the chondrocytes themselves (Pelletier *et al.*, 1993), suggesting that cartilage is responsible for its own destruction during the disease process. Cartilage has been shown to degrade itself via stimulation with retinoic acid (Kistler *et al.*, 1991).

Cytokines are the mediators of connective tissue destruction in arthritic conditions (Pelletier *et al.*, 1993). Cytokines. in particular IL-1, are capable of causing chondrocytes to degrade their own ECM and are likely to play a role in OA (Carney, 1991). Several studies have investigated the effects of cytokines on cartilage *in-vivo* (van Beuningen *et al.*, 1993; van de Loo *et al.*, 1995), *in-vitro* (Martell-Pelletier *et al.*, 1991; Mort *et al.*, 1993; van Beuningen *et al.*, 1993; Cawston *et al.*, 1994), and on chondrocytes *in-vitro* (Martell-Pelletier *et al.*, 1991; Chandrasekhar *et al.*, 1993; Shingu *et al.*, 1993; Gunther *et al.*, 1994).

Many of the studies centre on the effects of IL-1, which has been generally shown to stimulate proteoglycan degradation (van Beuningen *et al.*, 1993) whilst suppressing proteoglycan synthesis (van de Loo *et al.*, 1995). It has also been shown to stimulate collagen degradation (Cawston *et al.*, 1994) and MMP activity (Martell-Pelletier *et al.*, 1991; Chandresekhar *et al.*, 1993; Shingu *et al.*, 1993) while either decreasing (Martell-Pelletier *et al.*, 1993) or increasing (Shingu *et al*, 1993) TIMP production. TNF-α may have similar effects (Pelletier *et al.*, 1993; van de Loo *et al.*, 1995).

A number of growth factors, particularly the TGF-β family and IGF-1 have been shown to counter the effects of IL-1 (Chandresekhar *et al.*, 1993; van Beuningen *et al.*, 1993; Gunther *et al.*, 1994), as have antibodies to IL-1 (van de Loo *et al.*, 1995). There is, therefore, a complex level of control governing the actions of MMPs, TIMPs and cartilage matrix destruction. Although much is known about the effects and interactions of certain cytokines and growth factors, little data is available on the overall driving force and interactions of

these factors as a whole (see Pelletier *et al.*, (1993) for a comprehensive overview of the role and effects of cytokines on cartilage).

## 1.6. The Neovascularisation of Cartilage:

Neovascularisation rarely occurs in the adult (Moses, 1993). However, a number of conditions exist in which abnormal angiogenesis is an important element, including tumour growth, psoriasis, wound repair, rheumatoid arthritis and duodenal ulcers (Folkman, 1995).

Articular cartilage is regarded as an avascular tissue (Hamerman, 1989; Poole *et al.*, 1993). The avascular nature is not clearly understood though it is generally considered to be a function of anti-angiogenic molecules within the tissue (Moses *et al.*, 1990; Moses, 1993). A specific angiogenic inhibitor, named cartilage derived inhibitor (CDI), has been isolated and shown to inhibit the proliferation and migration of capillary endothelial cells *in-vitro*, and angiogenesis *in-vivo* (Moses *et al*, 1990).

The concept of neovascularisation in OA cartilage is relatively new (Mankin 1974b; Jeffery 1994). It is thought important in three steps of OA pathology, the formation and growth of osteophytes, the advancement of the underlying subchondral bone and the remodelling of the joint (Brown & Weiss, 1988). The process is extremely complex and probably involves cartilage mineralisation, changes in protease activity and changes in inhibition of proteases (Reddi & Kuettner, 1981; Brown & Weiss, 1988, Moses, 1993). It is also possible that there is release of angiogenic factors encouraging

vascularisation to occur, and indeed a very low molecular weight substance (600 daltons) from cartilage, termed endothelial cell stimulating angiogenic factor (ESAF), has been described to stimulate vascularisation, and also to activate latent MMPs in cartilage (Brown & Weiss, 1988).

A pre-requisite of the OA process is the re-initiation of cartilage growth and mineralisation (Brown & Weiss, 1988). Un-mineralised cartilage is resistant to vascularisation, primarily due to endogenous protease inhibitors, though with the advent of calcification, there is a decrease in the activity of these inhibitors, with a concomitant increase in protease activity (Reddi & Kuettner, 1981). Proteases can be produced by actively migrating endothelial cells (Heron *et al.*, 1986a & b; Fisher *et al.*, 1994) as well as chondrocytes (Granda *et al.*, 1990; Chandrasekhar *et al.*, 1993, Shingu *et al.*, 1993). As discussed earlier, there is an imbalance between the inhibitors and proteases in OA cartilage. The addition of more protease activity from invading vasculature can only contribute to the already occurring damage.

Although many of the mechanisms occurring within the cartilage and the chondrocytes during OA have been elucidated, the underlying reason for these degenerative changes, and ultimate loss of the cartilage, is still unknown.

## 1.7 Aims of the Project:

The aim of the project is to study cartilage vascularisation, with particular focus on the effect of endothelium on the chondrocytes and collagens of cartilage.

More specifically, *in-vivo* and *in-vitro* models of cartilage vascularisation were employed to specifically determine:

i) Is all developing hypertrophic cartilage able to initiate vascularisation, and is there a temporal and spatial control of the invasion?

ii) Is there specific interaction between developing hypertrophic chondrocytes and the endothelium?

iii) Is there a change in collagen expression during vascularisation of developing hypertrophic cartilage?

iv) What is the ultimate fate of developing hypertrophic chondrocytes undergoing vascularisation?

v) Does human OA cartilage lose its anti-angiogenicity and become able to allow vascular invasion?

It is hoped that the work with the developing hypertrophic cartilage i.e. cartilage that inherently becomes vascularised, will serve as a model for the effects of the vasculature on chondrocytes and matrix during pathological vascularisation of cartilage as seen in OA.

# CHAPTER 2

# Intrinsic Control of Vascularisation in Developing Cartilage Rudiments

(Part published in 'International Journal of Experimental Pathology', **78**, 187-196)

## 2.1. INTRODUCTION

At a defined time during development, hypertrophic cartilage is invaded by vascular tissue and erosion begins (Roach & Shearer, 1989; Price *et al.*, 1994). As resorption proceeds, the epiphyseal cartilage is gradually replaced by bone until only a thin layer remains at the articular surface (Price *et al.*, 1994). Although the general process of endochondral ossification is similar in birds and mammals, avian long bone development differs from that in mammals in that a larger proportion of the cartilage is removed prior to ECM calcification and the resorbed tissue is mainly replaced with a larger bone marrow cavity. There is, therefore, a lack of true endochondral ossification in avian bone development, except at the extremities of the diaphysis (Fell, 1925).

Due to the ease of obtaining specimens and the accuracy to which development can be staged, many studies have been performed on developing chick embryo cartilage (Fell, 1925; Silvestrini *et al.*, 1979; Rooney, 1984; Carrino & Caplan, 1985; Pechak *et al.*, 1986a & b; Roach & Shearer, 1989; Kaji *et al.*, 1990; Galotto *et al.*, 1994; Gibson *et al.*, 1995a & b; Roach *et al.*, 1995). Hypertrophy of the central zone chondrocytes begins, in the presumptive femur, at Hamburger and Hamilton stage 30 (6 days) (Hamburger and Hamilton, 1951; Rooney, 1984; Rooney *et al.*, 1984; Pechak *et al.*, 1986b). Between stages 32 and 34 (7-8 days) there is a progression of hypertrophy of the chondrocytes, as determined by an increase in cell size, loss of cell density and increased vacuolation (Rooney *et al.*, 1984; Pechak *et al.*, 1986). In the leg rudiments at stage 35 (9 days), vascular elements and associated cells invade

the hypertrophic core at a central point (Fell, 1925; Rooney & Kumar, 1993). By stage 36 (10 days), invasion is such that the central section of the hypertrophic cartilage has been eroded and the marrow cavity is forming (Pechak *et al.*, 1986a & b). This process occurs at a slightly later time in the wing bone rudiments (Rooney, 1984).

Approximately five days after the initiation of diaphyseal vascularisation, medullary spaces begin to form in the epiphyseal cartilage containing rounded cells. Although diaphyseal vascularisation is continuing, the invading epiphyseal vessels are quite separate (Fell, 1925). Eventually, expansion of both ossification centres leaves a narrow belt of cartilage, the epiphyseal growth plate (Fell, 1925).

Vascular invasion always begins within the central hypertrophic cell region. The control of this process is not well understood. The aims of this study were to determine whether all hypertrophic cells can stimulate or allow vascularisation and whether the process is temporally controlled. In addition the role the periosteum plays in the vascularisation process was examined. To do this, we have used chick embryo cartilage rudiments and the well-established model of the chick chorioallantoic membrane (CAM) to provide an *in-vivo* model of vascularisation.

## 2.2. MATERIALS AND METHODS

Eggs, rounded cell and hypertrophic cell cartilage, and BAEC were prepared as described (Appendix I).

*Grafting of Intact Rudiments*

Intact stage 32, 33, 34, 35 and stage 36 rudiments (approximately 7, $7^1/_2$, 8, 9 and 10 days respectively) were grafted immediately onto the CAM of 10 day hosts. The eggs were sealed and returned to the incubator for a further 3 days when the CAM surrounding the grafted tissue was excised, fixed in 10% buffered formalin and processed for wax histology. Three rudiments of each stage were added to each CAM and a minimum of five eggs for each stage of rudiment were used, i.e. at least 15 rudiments of each stage were grafted.

In a separate series of experiments, intact stage 32 and stage 36 rudiments were grown in organ culture in BGJb Fitton-Jackson modified medium, supplemented with 10% foetal bovine serum, L-glutamine (1.4 mM), Penicillin (50 IU/ml) and Streptomycin (50 µg/ml) (all Gibco), for 3 days prior to CAM grafting for a further 3 days. At the end of the graft period, tissues were fixed and processed as above.

*Grafting of Hypertrophic Zones*

Hypertrophic cell zones were isolated from leg rudiments at each stage between stages 32 and 36 and grafted onto the CAM of 10 day host embryos. Three zones were grafted onto each CAM and a minimum of 8 eggs were used for

each starting stage of the rudiment i.e. at least 24 hypertrophic zones of each stage were grafted. Samples of grafted hypertrophic zones were fixed at 12 hour intervals. The maximum time of grafting was such that for each starting stage, the total time, i.e. time of development *in-ovo* plus time on the CAM equaled 11 days (Table 2.1). Some individual hypertrophic zones were isolated, fixed and processed directly for histology to ensure the efficiency of isolation of hypertrophic cartilage.

**Table 2.1.** Table to show the time period of grafting of hypertrophic zones.

| **Age of hypertrophic zone *in situ*** | **Time on CAM (Days)** |
|---|---|
| Stage 32 (7 days) | 1-4 |
| Stage 34 (8 days) | 1-3 |
| Stage 35 (9 days) | 1-2 |
| Stage 36 (10 days) | 1 |

In one series of experiments, a detailed study of vascularisation was performed by removing and processing samples of stage 32 hypertrophic zones at 4 hours, 1 day, $1^1/_2$ days, 2 days, $2^1/_2$ days, 3 days and then at 4 hourly intervals until 5 days on the CAM.

In an attempt to determine the role played by the periosteum in vascularisation, periostea were removed from stage 34 (approximately 8 day) and stage 36 (approximately 10 day) hypertrophic zones prior to grafting for up to 4 days.

Removal of the periostea was achieved by carefully cutting along their

length with either a scalpel or by tearing the periosteum with a pair of sharp forceps and gently teasing the cartilage out of the opened zone.

To investigate the effect of enzymatic degradation on vascularisation, hypertrophic zones from stage 36 embryos, both with and without their periosteum, were pre-treated with either collagenase (Type 1A, 0.2% wt/vol., Sigma), or hyaluronidase (Type 1-S, 0.05% wt/vol., Sigma) or a combination of both in unsupplemented α-MEM medium for 20 minutes and then grafted onto the CAM for 2-3 days.

*Grafting of Rounded Zones*

Rounded zones were isolated from stage 36 rudiments and grafted onto the CAM of 10 day hosts. As above, three grafts were made per CAM and samples were fixed daily at intervals for up to 5 days.

In one series of experiments, rounded cell zones were pre-treated with collagenase and/or hyaluronidase as described above.

*Histological and Histochemical Examination*

Sections of CAM plus the grafts were cut at 5-7μm and general histological staining was performed using haemotoxylin & eosin. Various histochemical stains were also employed. These were safranin-O, toluidine blue and alcian blue at critical electrolyte concentrations (CEC) of 0.06M, 0.7M and 0.9M

$MgCl_2$ to identify alterations in proteoglycan and GAG composition (Appendix II).

*BAEC Monolayer Grafts*

BAEC were plated into 35mm dishes at $1x10^5$ cells per dish, and cultured until confluent, before use in grafting experiments. Hypertrophic zones were either grafted intact onto a monolayer of BAEC, or the periosteum was removed and this, along with the periosteum free hypertrophic cartilage were grafted individually onto separate monolayers.

## 2.3. RESULTS

*CAM Graft- Macroscopic Observations*

Despite being placed gently on the CAM, approximately 35% of the intact rudiments encouraged a slight leakage of blood around the graft site. Only 60% of the rudiments attached to the CAM and as observed by Thorogood (1983) the rudiments, instead of growing longitudinally, formed hoops and arches. A similar transition is seen when rudiments are maintained in organ culture.

The majority (approx. 70%) of the hypertrophic zones grafted encouraged a relatively large leakage of blood around the graft site when viewed after the first 24 hours irrespective of the *in situ* age of the zone (Figure 2.1). Zones removed at 4 hours had not adhered to the CAM, however, those removed after 24 hours were attached and were enveloped by the CAM. Blood vessels could often be seen growing over the cartilage.

Periosteum-free hypertrophic zones did not cause blood leakage, but still became enveloped in the CAM. Blood vessels were observed growing over and around the cartilage. Those treated with collagenase were no longer visible after 3 days on the CAM, and as such, no histological examination could be performed.

Rounded zone cartilage generally encouraged a small leakage of blood and was enveloped by the CAM, with blood vessels on and around the cartilage. This was also seen with enzymatically treated rounded cell zones.

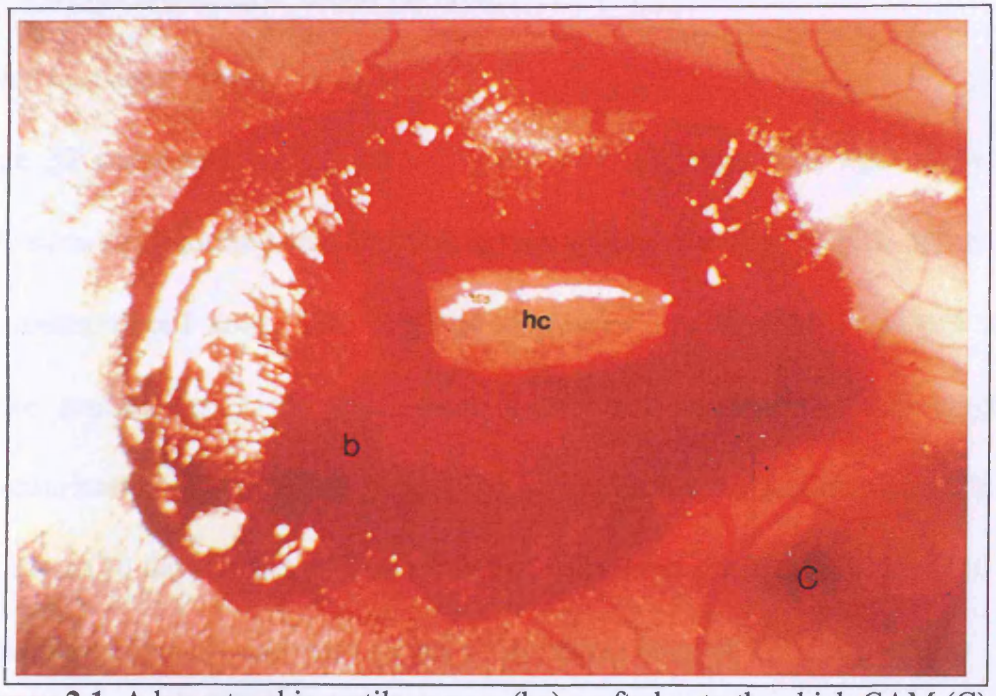



**Figure 2.1.** A hypertrophic cartilage zone (hc) grafted onto the chick CAM (C) for 36 hours. There is a large quantity of blood leakage (b) around the zone. This is never seen within the first 24 hours (x 25).

## *CAM Graft Histologic Observations*

### *Intact Rudiment Grafts*

Stage 32 rudiments grafted for 1-2$^1/_2$ days (8-9$^1/_2$ days total age) showed no indication of becoming vascularised, either within the hypertrophic cell zone or the rounded cell zone. The cartilage appeared healthy and remained intact. Those grafted for 3 days (10 days total age) showed the first stages of vascularisation within the hypertrophic zone. Stage 33 rudiments grafted for 2$^1/_2$ days (10 days total age) and stage 34 rudiments grafted for 2 days (10 days total age) showed the primary stages of invasion (Figure 2.2).

Stage 35 and 36 rudiments grafted for 3 days (12 and 13 days total age respectively) showed a pattern of vascularisation similar to that observed in stage 38 and 39 rudiments (12 and 13 days old respectively) *in-situ* i.e. most of the central portion of the hypertrophic zone was extensively eroded (Figure 2.3). The extent of invasion was greater than that seen in a stage 36 rudiment fixed immediately upon removal, indicating that invasion continued when the rudiment was grafted. The invasion site contained a mixture of cell types, including erythrocytes, fibroblastic cells and a number of cells containing darkly staining granules, or which were possibly multinucleated (Figure 2.4). In contrast the rounded cell zones of the rudiments showed no signs of vascular invasion during the 3 day culture period.

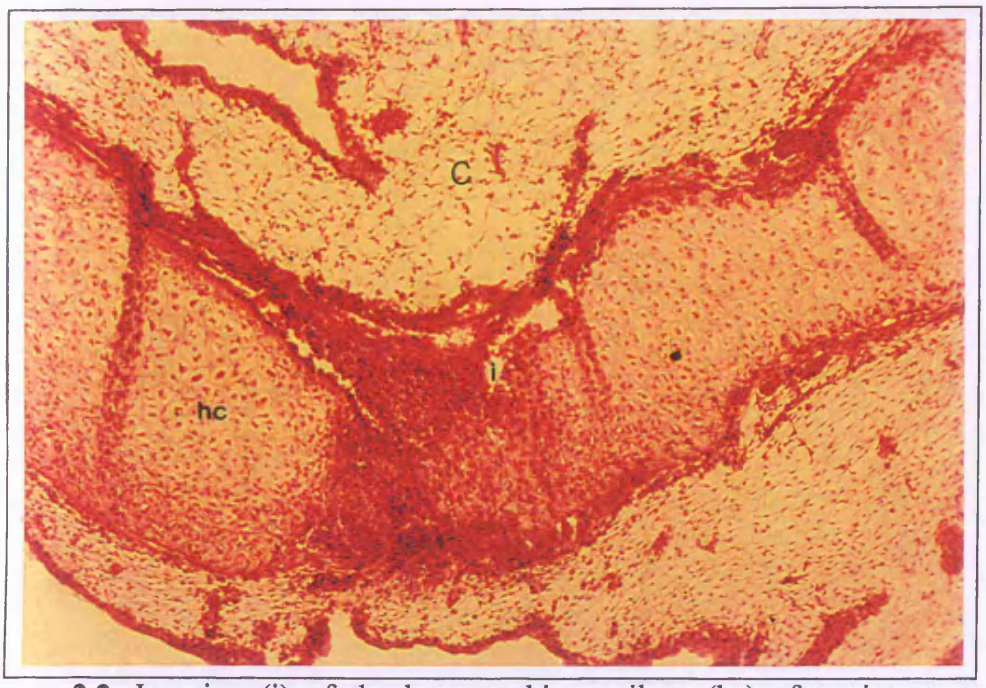



**Figure 2.2.** Invasion (i) of the hypertrophic cartilage (hc) of an intact stage 34 rudiment after being grafted on the CAM (C) for 2 days. The amount of erosion seen is similar to that seen in a normal stage 36 tibia. (H&E, x 100).

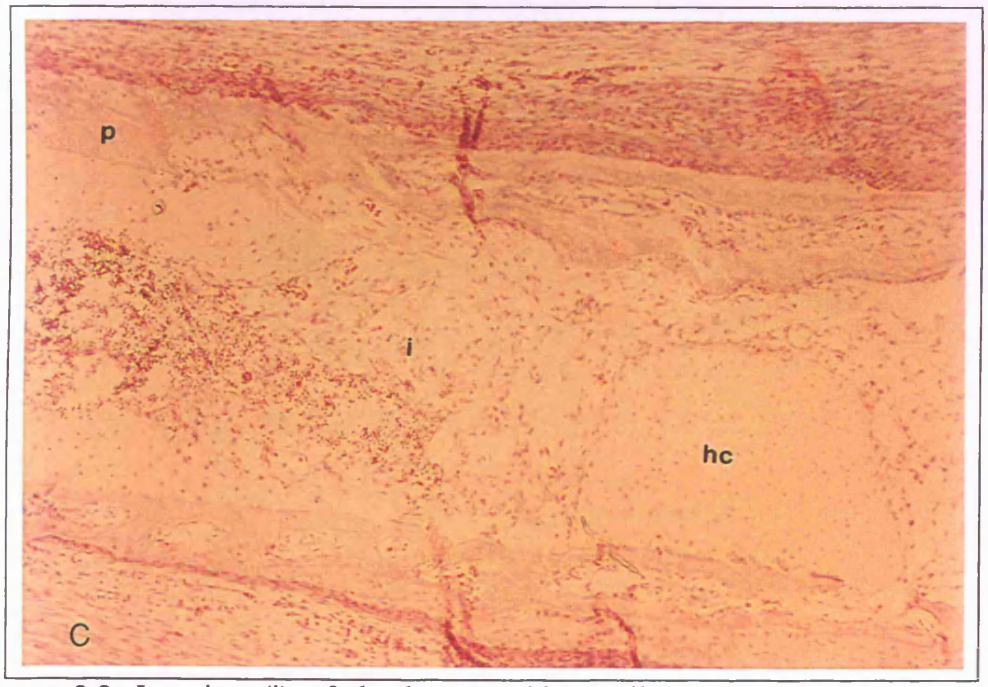



**Figure 2.3.** Invasion (i) of the hypertrophic cartilage (hc) of an intact stage 35 rudiment after being grafted on the CAM (C) for 3 days. The amount of erosion seen is similar to that observed in a normal stage 38 rudiment. p is the periosteum (H&E, x 200)

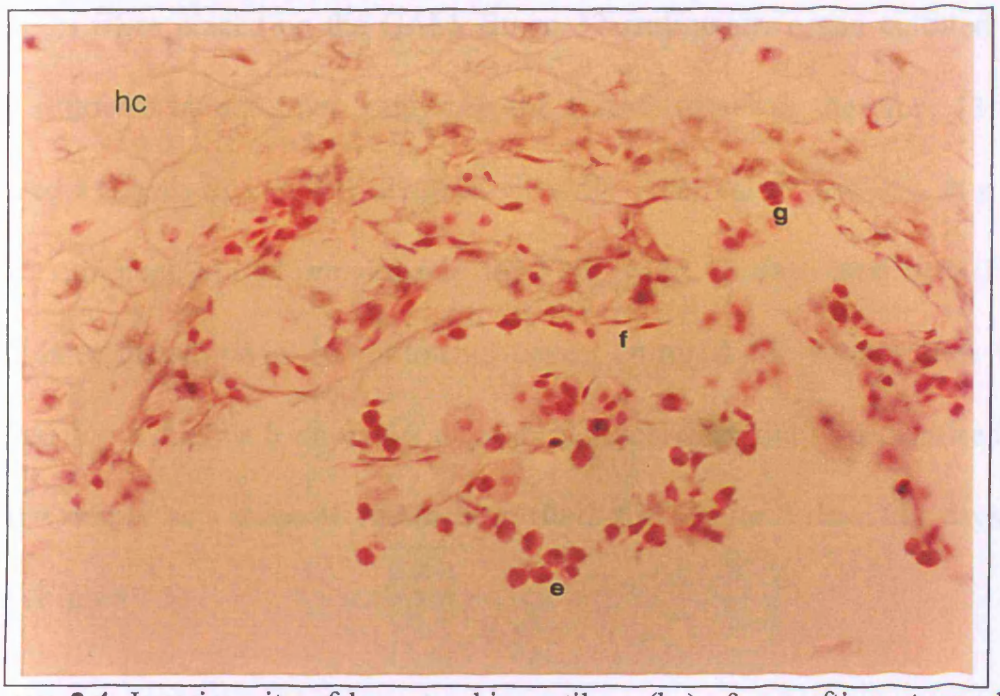
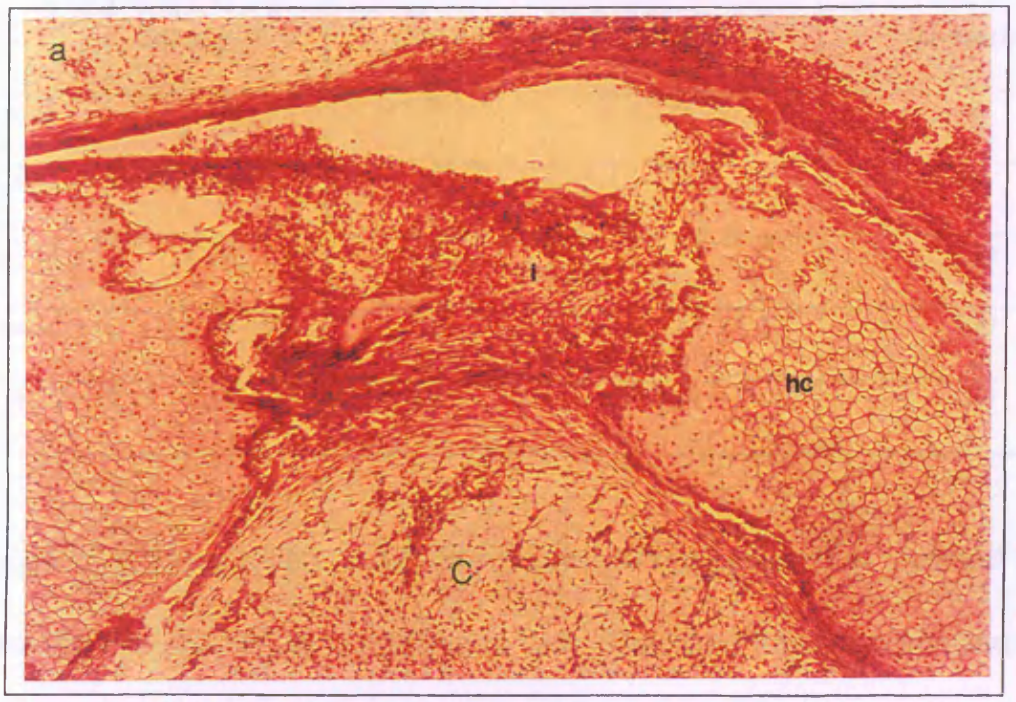



**Figure 2.4.** Invasion site of hypertrophic cartilage (hc) after grafting. A number of different cell types are present, including fibroblasts (f), erythrocytes (e) and a granular or possibly multinucleated cell (g). (H&E, x 400).

Rudiments grown in organ culture prior to CAM grafting also showed cellular invasion when placed on the CAM. Stage 32 rudiments, organ cultured for 3 days followed by a further 3 days on the CAM (total age therefore 13 days) showed a degree of vascular invasion virtually identical to that seen in a stage 35-36 rudiment grafted immediately for 3 days (12-13 days total age) (Figure 2.5). In contrast, stage 36 rudiments organ cultured for 3 days then CAM grafted for a further 3 days (16 days total age) were only vascularised to a similar degree as a stage 36 rudiment grafted directly for 3 days (13 days total age) (Figure 2.5).

*Hypertrophic Zone Grafts*

Histological staining using H&E of stage 32 hypertrophic zones grafted for between 0 and $2^{1}/_{2}$ days showed no sign of cartilage vascularisation. However, approximately 80% of stage 32 grafts removed after 3-5 days of incubation (total age of 10-12 days) exhibited vascular invasion (see Table 2.2.). All stage 34 zones grafted for 3 days and stage 35 zones grafted for 2 days showed varying degrees of vascularisation indicating that vascularisation began while the cartilage was on the CAM. Stage 36 zones grafted for 1 day also showed extensive vascularisation but a stage 36 zone may be partially vascularised prior to grafting due to the process beginning during stage 35 (Pechak et al, 1986b). Our own observations of stage 36 rudiments agree with these findings.



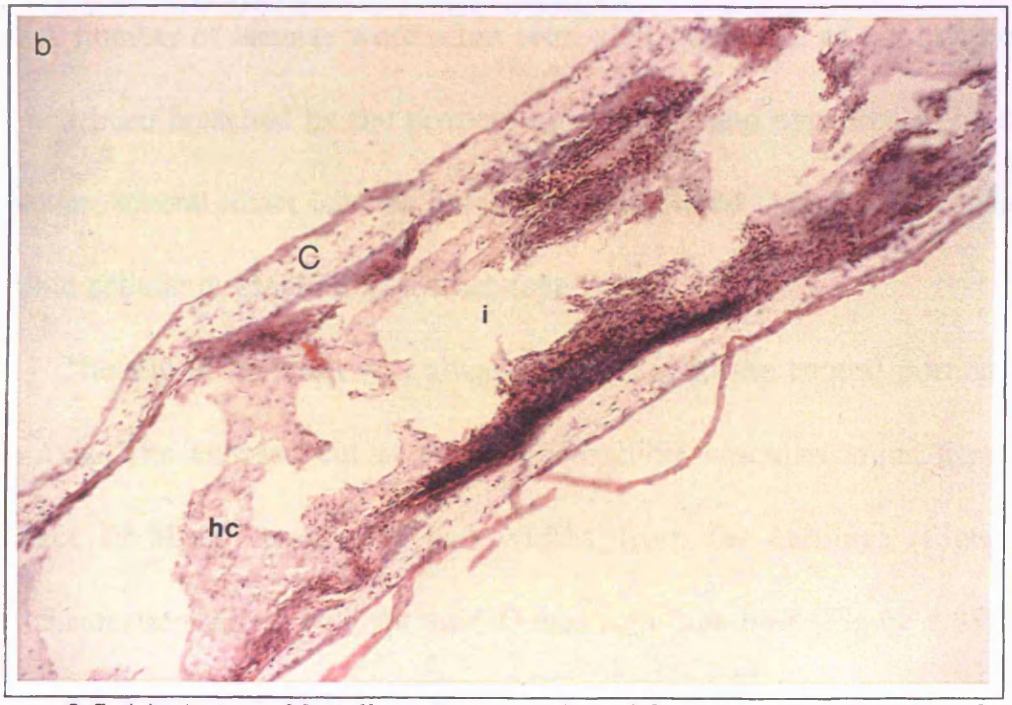




**Figure 2.5. (a).** A stage 32 rudiment organ cultured for 3 days then CAM grafted for a further 3 days. Most of the central portion of the hypertrophic cartilage (hc) has been invaded and eroded (i) similar to that seen in a stage 35/36 rudiment that has been grafted for 3 days. C is the CAM tissue (H&E, x100). **(b).** A stage 36 rudiment organ cultured for 3 days then grafted for a further 3 days. The extent of erosion is again similar to that seen in a stage 35/36 rudiment that has been grafted for 3 days. hc is the hypertrophic cartilage, i is the invaded area, C is the CAM. (H&E, x 100).

**Table 2.2.** Table to show the times at which isolated hypertrophic zones become vascularised on the CAM.

| Age of Zone | Number of zones grafted | Time on CAM (Days) | Total age of Zone (Days) | % of zones vascularised |
|---|---|---|---|---|
| Stage 32 | 30 | 0-2$^1/_2$ | 7-9$^1/_2$ | 0 |
| Stage 32 | 30 | 3-5 | 10-12 | 80 |
| Stage 34 | 24 | 3 | 11 | 100 |
| Stage 35 | 24 | 2 | 11 | 100 |
| Stage 36 | 24 | 1 | 11 | 100 |

The onset of hypertrophic cartilage erosion always occurred at a central point along the long axis of the zone (Figure 2.6). The site of invasion contained the same mixture of morphologically distinct cells as described earlier (see Figure 2.4). A number of lacunae were often seen along the edge of the invasion site that had been breached by the process of invasion and appeared to be empty. However, several intact lacunae contained vacuolated chondrocytes indicating possible cellular degeneration or death (see Figure 2.9).

The site of invasion was always restricted to the central portion of the long axis. The exposed cut ends did not exhibit vascularisation, despite the presence of blood vessels 1-2 cell widths from the cartilage (Figure 2.7). Metachromatic staining with safranin-O and toluidine blue (Figure 2.8) showed a loss of staining intensity in the cartilage along the leading edge of the invasion site indicating a loss of proteoglycans within the matrix. This loss of

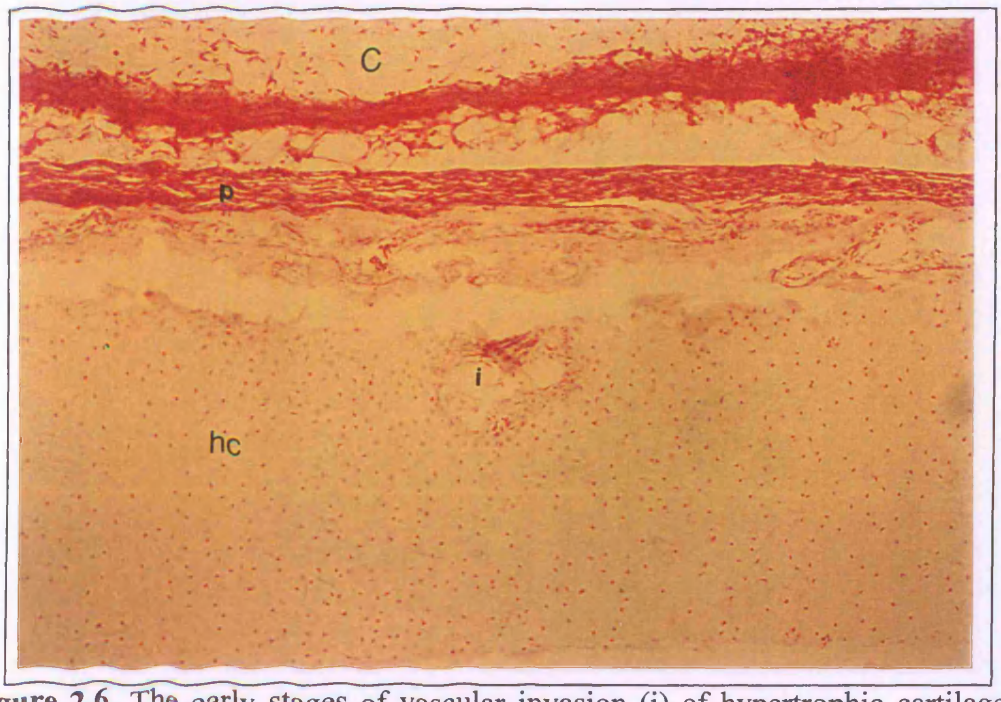



**Figure 2.6.** The early stages of vascular invasion (i) of hypertrophic cartilage (hc). This is always seen to occur at a central point along the axis of the zone. C is the CAM tissue, p is the periosteum. (H&E, x 200).

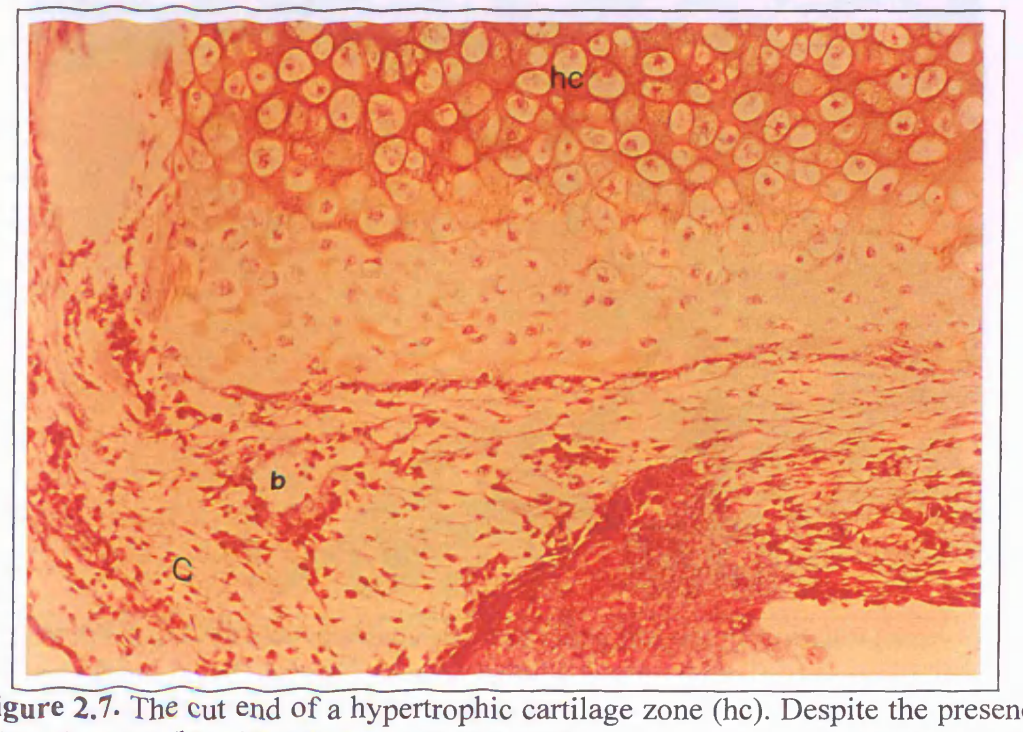



**Figure 2.7.** The cut end of a hypertrophic cartilage zone (hc). Despite the presence of a blood vessel (b) within 2-3 cell widths of the cartilage, there is no evidence of any vascular invasion. There is, however, a loss of metachromatic staining along the cut edge of the zone. (Safranin-O, x 400).

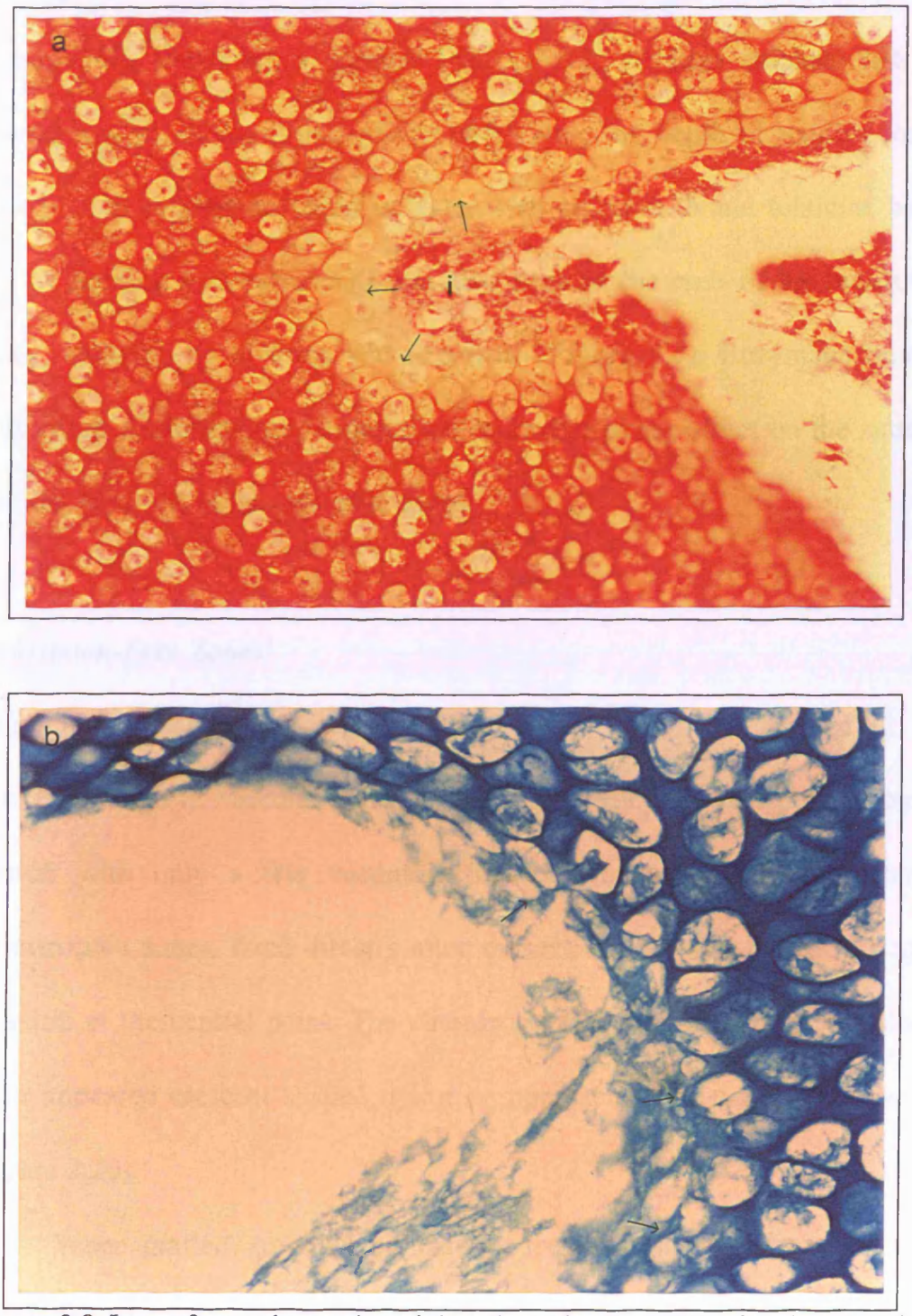



**Figure 2.8.** Loss of metachromatic staining along the rim of invasion (arrows). i is the invasion front. **(a).** Safranin-O loss of staining occurs up to 2 lacunae widths (x 400). **(b).** Toluidine blue, staining loss is only up to 1 lacunae width. (x 400).

staining only penetrated 1-2 lacunae widths into the cartilage. A similar loss of staining was seen with alcian blue CEC at 0.9M, 0.7M and 0.06M $MgCl_2$ (Figure 2.9), indicating a specific loss of KS, heparan sulphate (HS) and general loss of all cartilage GAG respectively. The depth of staining loss did not occur to as great an extent as seen with safranin-O and toluidine blue. A loss of staining with safranin-O was also seen at the ends of the hypertrophic zones, although no invasion had occurred (Figure 2.7). Enzymatic treatment with either hyaluronidase, collagenase or both had no effect on the pattern of vascular invasion of hypertrophic zones.

*Periosteum-Free Zones*

Stage 34 periosteum-free zones, grafted for 4 days (total age of 12 days) showed no sign of vascular invasion. The chondrocytes were morphologically normal with only a few vacuoles. Sections of stage 36 periosteum-free hypertrophic zones, fixed directly after dissection, showed the early stages of invasion at the central point. The chondrocytes contained many vacuoles and often appeared crescent shaped, lying compressed against their lacunae walls (Figure 2.10).

When grafted, stage 36 periosteum-free zones showed no evidence of active vascularisation on the CAM. At the end of the 3 day culture period, chondrocytes throughout the entire zone appeared more healthy than

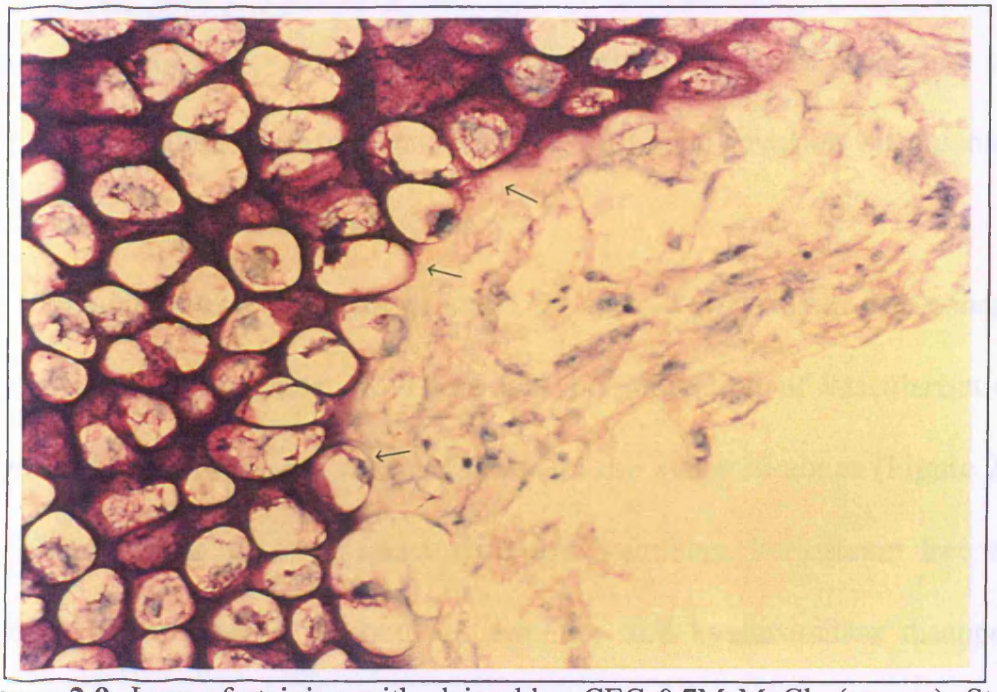


**Figure 2.9.** Loss of staining with alcian blue CEC 0.7M $MgCl_2$ (arrows). Staining loss occurs up to approximately 1 lacunae width. The stain is specific for heparan and keratan sulphate. Many of the chondrocytes around the invasion front appear vacuolar and degenerate. (x 400).

chondrocytes from non-grafted stage 36 hypertrophic regions, in that they showed a normal rounded chondrocytic phenotype and contained few vacuoles. Some of the chondrocytes were undergoing mitosis. A small proportion of the cells, which increased in concentration towards the invasion site, contained darkly staining granules (Figure 2.10).

Hyaluronidase treatment of stage 34 and 36 periosteum free zones had little effect on vascularisation. There was no indication of vascularisation at stage 34, or any continued vascularisation in the stage 36 zones (Figure 2.11). The chondrocytes appeared intact with few vacuoles. Periosteum free zones treated with collagenase or both collagenase and hyaluronidase disappeared while on the CAM during the 3 day culture period and consequently no histological examination could be performed.

*Rounded Cell Zones*

Stage 36 zones grafted for 3 days showed no sign of vascular invasion. The cells were healthy and the cartilage was intact (Figure 2.12). Zones grafted for 5 days showed the first stages of vascular invasion (Figure 2.13), similar to that seen in cartilage canal formation *in-situ*. One or two small vessels appeared within the rounded cell cartilage containing a similar mix of cells seen during hypertrophic zone invasion.

Enzymatic treatment of stage 36 rounded cell zones caused a change in the pattern of vascularisation. Hyaluronidase treated zones became invaded in

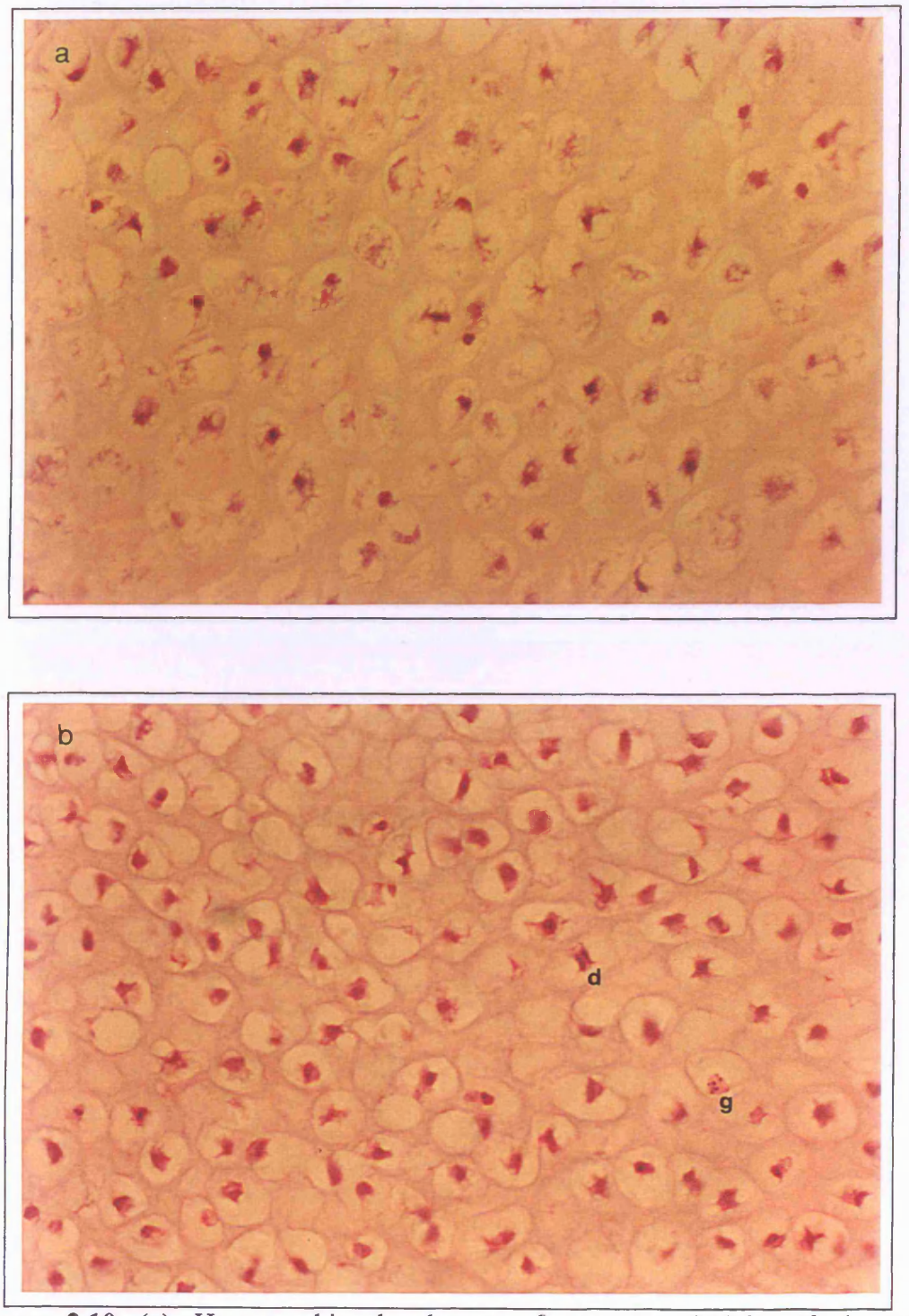



**Figure 2.10. (a).** Hypertrophic chondrocytes from a non-CAM grafted stage 36 hypertrophic zone. The cells are very vacuolar and are morphologically degenerate. (H&E, x 400). **(b).** Hypertrophic chondrocytes from a CAM grafted periosteum free stage 36 hypertrophic zone. The cells do not appear morphologically degenerate, the occassional cell is even undergoing mitosis (d). A number of cells contain dark granules (g). (H&E, x 400)

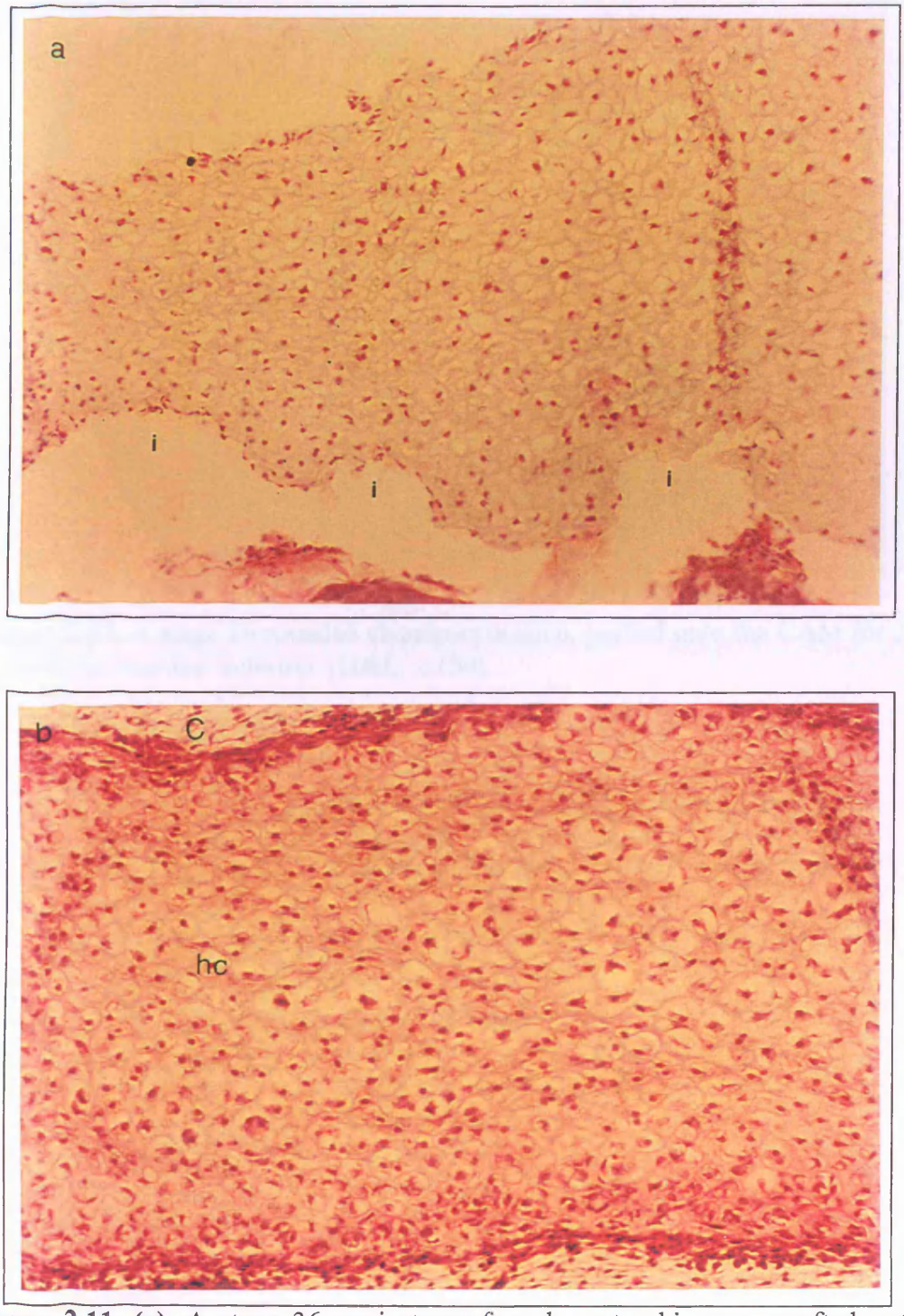



**Figure 2.11. (a).** A stage 36, periosteum free, hypertrophic zone grafted onto the CAM for 3 days. i's indicate possible initial invasion sites before the periosteum was removed. (H&E, x 200). **(b).** A hyaluronidase treated, periosteum free, stage 34 hypertrophic cartilage zone (hc), grafted onto the CAM (C) for 3 days. No indication of vascular invasion can be seen. (H&E, x 200).

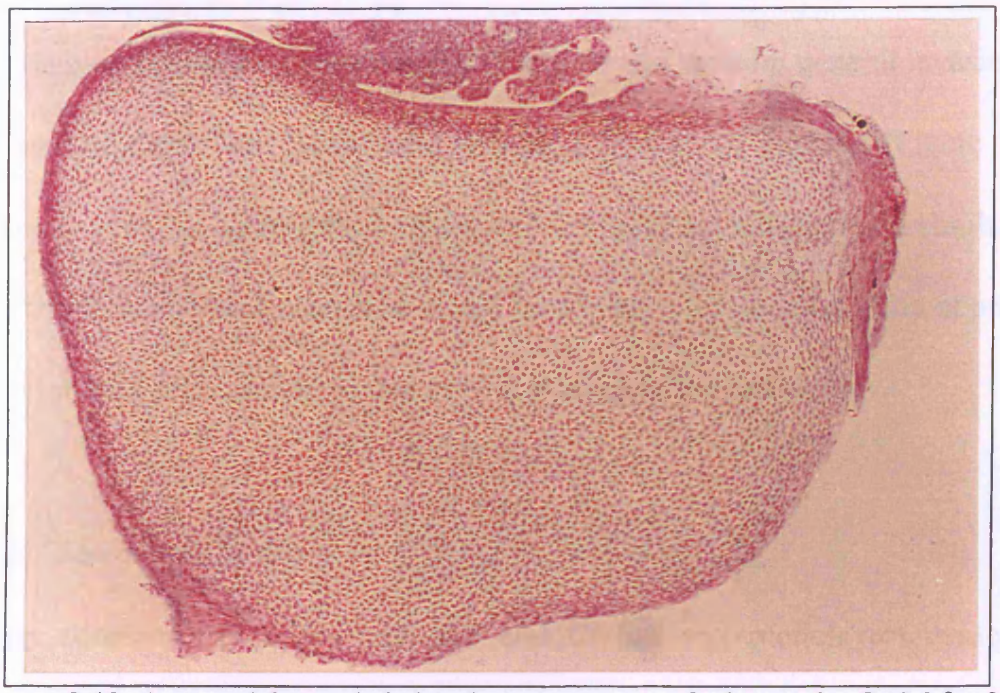
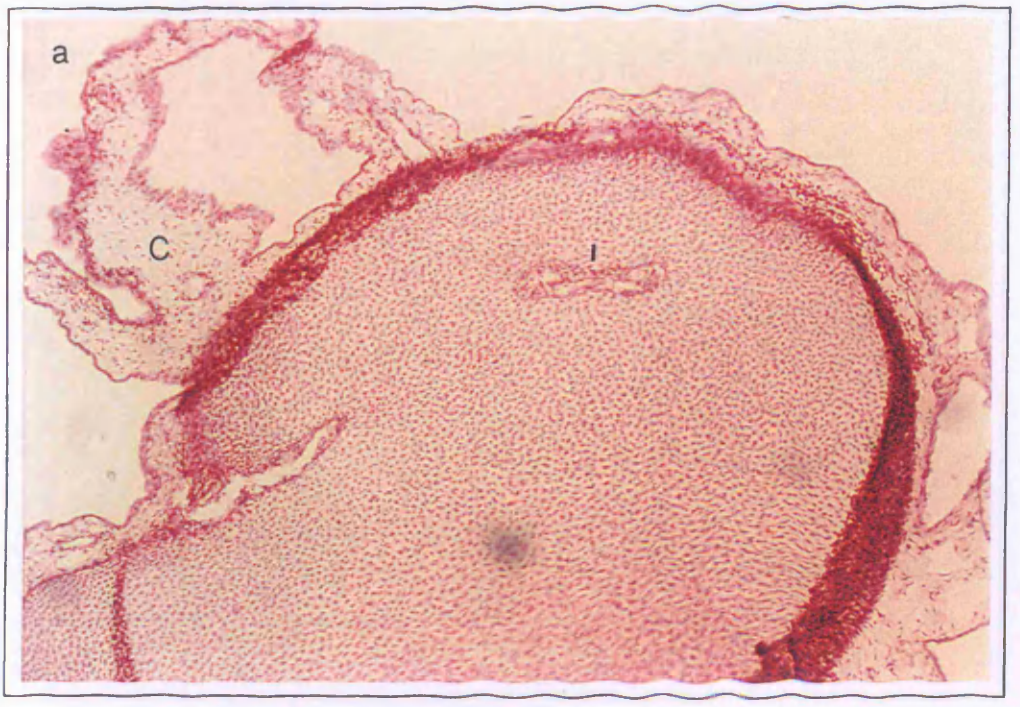


**Figure 2.12.** A stage 36 rounded chondrocyte zone, grafted onto the CAM for 3 days. There is no vascular invasion. (H&E, x 100).

an identical way to untreated zones grafted for 5 days as seen in Figure 2.13, except that the invasion occurs within 2-3 days of grafting. Treatment with collagenase caused extensive tissue disruption and random general invasion and erosion of the rounded cell zone within 2-3 days of grafting (Figure 2.13). Zones exposed to both hyaluronidase and collagenase behaved in a similar way to those treated with collagenase alone, however, occasionally, a site of primary invasion was seen, similar to that in hyaluronidase treated zones.

*BAEC Monolayer Grafts*

Intact zones, when placed onto the endothelial cell monolayers caused the formation of endothelial cell tubules, above the cobblestone monolayer of endothelial cells, normally around the cut ends of the zone, within 1-2 days of culture (Figure 2.14). Individual periostea, stripped off from the cartilage had an identical effect on the monolayer (Figure 2.14). The formation of tubules was only seen in the near vicinity of the tissue and not throughout the dish. However, the hypertrophic cartilage that was removed from inside the periosteum did not cause formation of endothelial cell tubules during the culture period (Figure 2.15).



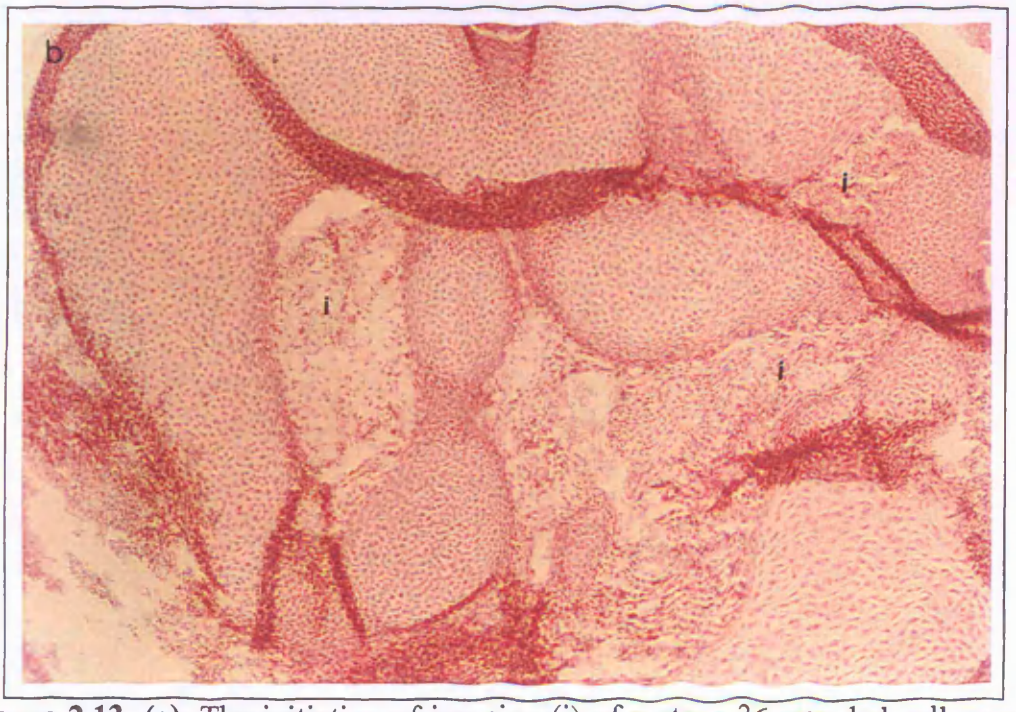




**Figure 2.13. (a).** The initiation of invasion (i) of a stage 36 rounded cell zone after being CAM grafted for 5 days. A similar pattern is seen in rounded cell zones after hyaluronidase treatment, though at an earlier time. C is the CAM. (H&E, x 100). **(b).** Invasion of a single stage 36 rounded cell zone after treatment with collagenase and CAM grafting for 3 days. i's indicate areas of invasion and erosion. (H&E, x 100).

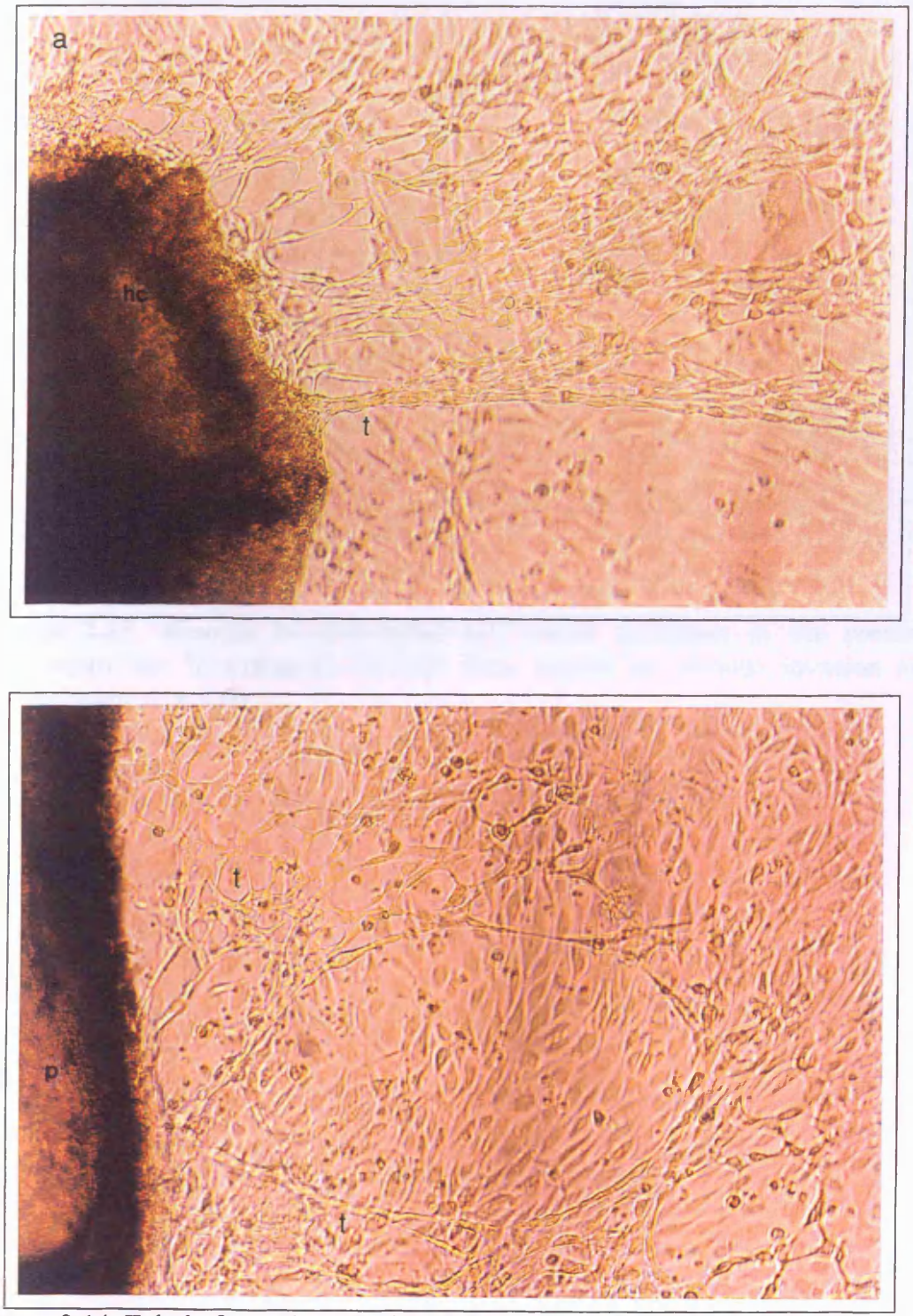



**Figure 2.14.** Tubule formation (t) of BAEC in culture, induced by the addition of **(a).** an intact hypertrophic zone (hc), and **(b).** the periosteum (p) removed from the hypertrophic cartilage viewed under phase contrast. (x 100)

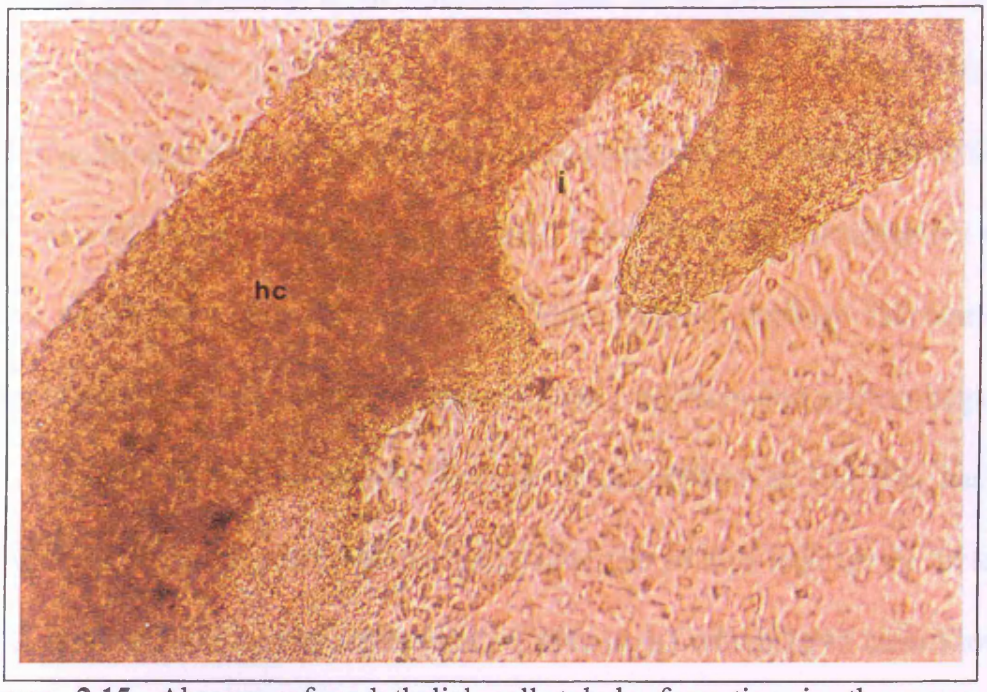



**Figure 2.15.** Absence of endothelial cell tubule formation in the presence of periosteum-free hypertrophic cartilage (hc), despite an obvious invasion site (i). (Phase contrast, x 100)

## 2.4. DISCUSSION

Cartilage vascularisation is an important step in the formation of bone. The results show that the temporal and spatial pattern of the invasion of hypertrophic and rounded cell cartilage is maintained even when the rudiments are removed from the embryo and grafted onto the CAM. Irrespective of the age at which the rudiments were taken, hypertrophic cartilage vascularisation always occurred at a total age of approximately 10 days and always began at a central point, as is seen *in-ovo* (Pechak *et al.*, 1986a & b). This has been reported to be due to a decrease in the production of anti-angiogenic factors by the embryonic cartilage (Pechak *et al.*, 1986a & b) which have been shown to be produced by the hypertrophic core (Sorgente *et al.*, 1975).

This result is similar to those obtained by Gibson *et al.* (1995b) who found that sterna from 20 day chick embryos, when cultured on the CAM, were resorbed specifically at the primary ossification centre, suggesting that only a specific region of cartilage, at a specific time is able to become vascularised.

This is most clearly seen with isolated hypertrophic cartilage. Interestingly the ends of these zones expose large areas of hypertrophic cartilage, yet invasion still only occurs from a central point, never from the exposed ends. *In-ovo,* however, this cartilage is resorbed, once the invasion has worked its way from the centre to the extremities of the cartilage (Rooney & Kumar, 1993). It is suggested here that all hypertrophic cartilage has the capacity to become resorbed, but not all hypertrophic cartilage, at a given time, has the capability to trigger the resorption process.

Gibson *et al.*, (1995b) also showed that sterna from 18 day embryos did not become vascularised when grafted onto the CAM. It was suggested that only cartilage that has started to become resorbed will continue the process on the CAM. This suggestion disagrees with our own observations. We have shown here that hypertrophic cartilage, still 2-3 days away from the initiation of resorption, still becomes resorbed in an identical spatio-temporal manner when grafted.

It appears that only a small subset of hypertrophic chondrocytes may have an intrinsic mechanism that can trigger vascular invasion and subsequent resorption. This is most clearly demonstrated from rudiments which are first organ cultured and then CAM grafted. Organ culturing the rudiments allows normal development of the pre-vascularisation processes. However once at a certain stage, the process cannot continue any further without the external influences of invasive tissue e.g. the CAM. It is also apparent that the 'trigger' for invasion does not disappear when rudiments are organ cultured, but remains within the cartilage until the rudiment is placed in an environment conducive to vascular invasion.

Endothelial cells are thought to have a central role in the process of vascularisation (Roach & Shearer, 1989; Gibson *et al.*, 1995b). The first stage in vascular invasion occurs outside of the cartilage and within the periosteal bone (Silvestrini *et al.*, 1979; Pechak *et al.*, 1986a). The vascular cells move through a hole in the inner periosteal layer, which is thought to be formed by phagocytic cells (possibly osteoclasts) which resorb a restricted region of

this inner periosteal layer (Peck, 1987). The cells associated with the vasculature subsequently dissociate the underlying cartilage (Pechak *et al.*, 1986a). This implies that vasculature-associated cells are important in triggering the invasion and resorption process.

This mixture of cells, or possibly factors produced by cells within the periosteum, appear vital for the initiation and continuation of the vascularisation and resorption process. Removal of the periosteum prior to, or immediately after, the primary invasion prevents the resorption process occurring or continuing respectively. This provides further evidence that only a specific portion of hypertrophic cartilage can trigger invasion or can be triggered into becoming invaded. It also indicates that a periosteal specific mixture of cells is required in the initiation and early stages of invasion.

An important observation is that periosteum-free cartilage cells resist the degeneration processes induced by vascularisation when grafted onto the CAM. We postulate that removal of the periosteum removes part or all of the vascularisation 'signal' and in turn, chondrocytes which have begun to degenerate in response to, and in readiness for cartilage resorption, are able to recover and can continue to grow and divide.

Further evidence that the periosteum is an essential factor in hypertrophic cartilage vascularisation was shown by the ability of the periosteum alone to encourage endothelial cell tubule formation in a monolayer of endothelial cells. Hypertrophic cartilage alone could not cause the same effect, despite the presence of a centre of erosion at the central point of the

cartilage when placed onto the cell monolayer (Figure 2.14).

The role of the cartilage matrix was investigated by using rounded cell zones from Hamburger & Hamilton stage 36 embryos. In these zones vascularisation begins approximately five days after the hypertrophic zones, to form the secondary centre of ossification at approximately 15 days of age (Fell, 1925). When grafted onto the CAM, stage 36 rounded cell zones did not become vascularised until the fifth day of culture. Treatment with hyaluronidase allowed apparently normal vascular invasion to begin on the CAM after only 2 days. Treatment with collagenase, or both collagenase and hyaluronidase allowed much cellular invasion and resorption of the entire zone, again after only 2 days on the CAM. Insults to the integrity of the matrix therefore allowed more rapid invasion by the vasculature. In particular, hyaluronidase treatment allowed the normal invasion process to occur, but at an earlier time than would normally occur *in-ovo*, or if grafted without enzymatic treatment. It has been shown that breakdown products of hyaluronan are angiogenic (Rooney & Kumar, 1993). This suggests that damage to the matrix components is a primary step in cartilage invasion, and it is known that chondrocytes do have the ability to degrade the proteoglycan component of the matrix (Gibson *et al.*, 1995b). It is also believed that endothelial cells can initiate cartilage resorption via the release of proteases (Roach & Shearer, 1989).

In conclusion, hypertrophic cartilage zones from developing long bone cartilage rudiments in the chick embryo become vascularised in a pre-

defined temporal and spatial manner, even when removed from *in-ovo* and placed onto the CAM. We postulate an intrinsic mechanism within the hypertrophic cartilage as to when and where it becomes vascularised, which at least partly relies on cells present within the periosteum and on the integrity of the matrix.

# CHAPTER 3

# An *In-Vitro* Model of Cartilage Vascularisation

## 3.1. INTRODUCTION

There are conflicting views as to the nature of the initiating factor of vascular invasion of the hypertrophic cartilage core during development. Some believe a change in the cartilage occurs whereby there is a decrease in the levels of an anti-angiogenic substance, which, once below a threshold level, cannot prevent vascular invasion (Pechak *et al.*, 1986a). Others perpetuate a central role for the invading vascular elements, suggesting that factors arising from the endothelium are required to activate chondrocyte mediated degradation of surrounding cartilage matrix (Gibson *et al.*, 1995b). Further, recent work by Alini *et al.*, (1996) has shown the production of an angiogenic molecule by bovine hypertrophic cartilage, suggesting that hypertrophic cartilage may have the ability to induce vascularisation.

Developmentally, a great deal of work has been done on the chick embryo, (Fell, 1925; Silvestrini *et al.*, 1979; Pechak *et al.*, 1986a & b; Roach & Shearer, 1989; Galotto *et al.*, 1994; Gibson *et al.*, 1995a & b) and the rat (Reddi & Kuettner, 1981; Lewinson & Silbermann, 1992; DeSimone & Reddi, 1992). Despite this, there is still much debate about the chemical factors involved in triggering the cartilage invasion process.

Described here is an *in-vitro* co-culture model between chick embryo hypertrophic chondrocytes and bovine endothelial cells, to study the effect that

## 3.2. MATERIALS AND METHODS

Hypertrophic chondrocytes, BAEC and endothelial cell conditioned medium (ECCM) were prepared as described (Appendix I).

*Cell Culture*

All cultures were maintained at 37°C in 95% air, 5% $CO_2$ and were fed every second day with the appropriate medium. Cells were cultured under 5 culture conditions as follows:

i) Chondrocytes were plated as high density micromass colonies ($5x10^6$-$1x10^7$ cells/ml) in 10µl drops similar to Ahrens *et al.*, (1977). 2 micromasses were plated per 35mm dish and medium was pipetted around the edge of the dish to prevent cells drying out. These were allowed to settle overnight before the dishes were flooded with fresh medium and allowed to grow for another 3-5 days. After this initial period, $1x10^5$ BAEC were added to each dish.

ii) Single chick hypertrophic micromasses were plated with between 1 and 4 high density BAEC micromasses in 35mm dishes. Once the cells settled overnight, the dishes were flooded with fresh medium.

iii) Chondrocytes were plated as micromasses, one per well, into 24 well plates, allowed to settle overnight before flooding. The micromasses were fed with varying concentrations of ECCM from confluent cells diluted with

normal medium to a final concentration of 20, 40, 60, 80 and 100% for up to three weeks.

iv) Chondrocytes were plated into 35mm dishes (Nunc) at $1x10^5$ cells/dish. They were allowed to settle overnight before being fed with either fresh control medium or 100% ECCM from either confluent or sub-confluent endothelial cells. Some dishes had the ECCM removed after 4, 8, 12, 24 or 48 hours, which was replaced with fresh control medium.

v) Chondrocytes were plated as in iv) but over a thin layer of 2% agarose gel (ICN). The cells were maintained in normal medium overnight, before removal and replacement with 100% ECCM. Controls were fed with normal medium.

*Cell Counts:*

Cells plated at $1x10^5$ cells per dish and treated with control medium or 100% ECCM were removed with 1x trypsin/EDTA solution (0.05% trypsin, 0.53mM EDTA, Gibco), pelleted and resuspended in 1ml of phosphate buffered saline (PBS). Equal volumes of cell suspension and trypan blue were mixed and the number of living and dead cells per dish counted on a haemocytometer. The mean of a minimum of four dishes was recorded. An independent sample T-test was performed on the values to determine whether differences in cell number were significant.

*Alcian Blue Binding Assay:*

Performed according to Zimmerman *et al.*. (1994) on cultures from various timepoints. Cultures were rinsed with PBS before elution overnight in 0.6N HCl. The acid was removed and the cultures stained with 0.05% Alcian Blue in 3% acetic acid overnight before being rinsed in 5% acetic acid for 2 hours. Stained matrix components were eluted overnight in 4M GuCl (Sigma). The resultant solution was diluted 1 in 3 with 4M GuCl and the absorbance measured in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech) at 620nm. The results of the ECCM cultures are expressed as a percentage of the control cultures, the control value being 100% at each time point (Zimmerman *et al.*, 1994).

*Bromo-deoxy Uridine (BrdU) Labelling*

At various stages of culture, 0.01M 5'bromo-2-deoxy uridine (Sigma) was added to the culture medium and cultures incubated for 3 hours. The cells were then rinsed in PBS and fixed in 95% ethanol at -20°C for 15 minutes. After 2 further rinses in PBS, the cells were incubated in 2M HCl for 30 minutes at 37°C. 0.1M sodium tetraborate (Borax) (Sigma) pH 8.5 was used to neutralise the cultures for 10 minutes before a further 2 rinses in PBS. Dishes were then flooded with normal rabbit serum (Serotec) at 1:5 dilution with PBS for 10 minutes and the surplus removed. Monoclonal mouse anti-BrdU antibody

(monoclonal no. BU-33, Sigma) was added at 1:1000 in PBS for 1 hour at room temperature. Cultures were then rinsed in tris buffered saline (TBS) and a biotinylated rabbit anti-mouse antibody (Dako) was added at 1:200 dilution in PBS for 30 minutes at room temperature. After rinsing in TBS, cultures were incubated for 30 minutes with ABComplex (Dako) made up as per manufacturers instructions in 0.05M Tris/HCl (pH 7.6), cells rinsed again then incubated with DAB/$H_2O_2$ (Sigma Fast DAB Tablet, Sigma) for 5-10 minutes, rinsed and mounted in glycerine jelly. The numbers of positive and negative cells in 4 random areas of 0.8mm$^2$ in each dish were counted. The numbers of positive cells were expressed as a percentage of total number of cells counted.

*Culture of Other Cells in Conditioned Medium*

Other cell types were cultured with 100% ECCM. Cells used were chick rounded chondrocytes from 10 day embryonic rudiments, chick embryo skin and muscle fibroblasts, and human articular chondrocytes from OA knee cartilage. Chick rounded chondrocytes were prepared in the same fashion as hypertrophic chondrocytes, chick skin and muscle fibroblasts were prepared by digesting embryo skin and muscle tissue in 1mg/ml collagenase (Type 1A, Sigma) for 2-3 hours. Human chondrocytes were prepared by digesting finely diced articular cartilage in 2mg/ml collagenase (Type 1A, Sigma), for 4-6 hours. All cell types were plated at 1x10$^5$ cells per dish and settled overnight.

## 3.3. RESULTS

i) Hypertrophic chondrocyte micromasses with BAEC:

Chondrocyte micromasses settled overnight to give small mounds of rounded cells with a very refractive ECM (Figure 3.1). When added, the BAEC settled around the chondrocyte micromass no differently from control endothelial cells, though none were observed to settle on top of the chondrocytes themselves. Once the endothelial cells became confluent, the chondrocytes began to degenerate. The micromasses lost their three dimensionality and became monolayers of polygonal cells with a distinct lack of ECM compared to control chondrocytes. Within 1-2 days the cells became granulated and/or vacuolated (Figure 3.2). Columns of endothelial cells could be seen growing into the monolayer of chondrocytes, removing them from the base of the dish (Figure 3.3). By 5-6 days, all of the chondrocytes within the colony had disappeared, leaving a morphologically normal monolayer of endothelial cells. All of the chondrocytes throughout the micromass became degenerate at the same stage, not just those in contact with the endothelial cells.

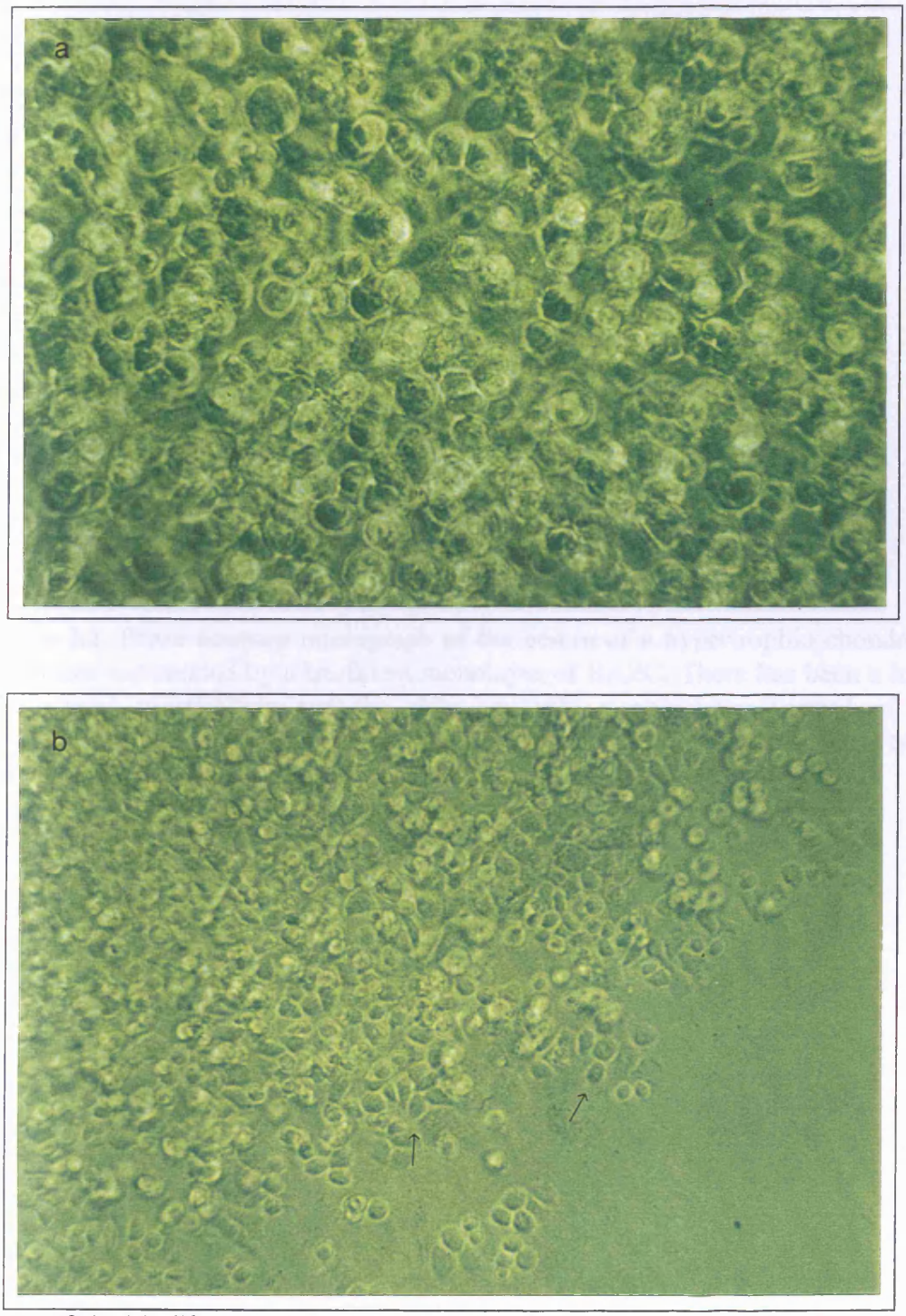



**Figure 3.1. (a).** Phase contrast micrograph of the central area of a hypertrophic chondrocyte micromass. The cells are rounded and show a refractive extracellular matrix (ECM). (x 200). **(b).** The edge of a hypertrophic chondrocyte micromass, showing a number of settled polygonal cells (arrows) with refractive ECM. (Phase contrast, x 100).

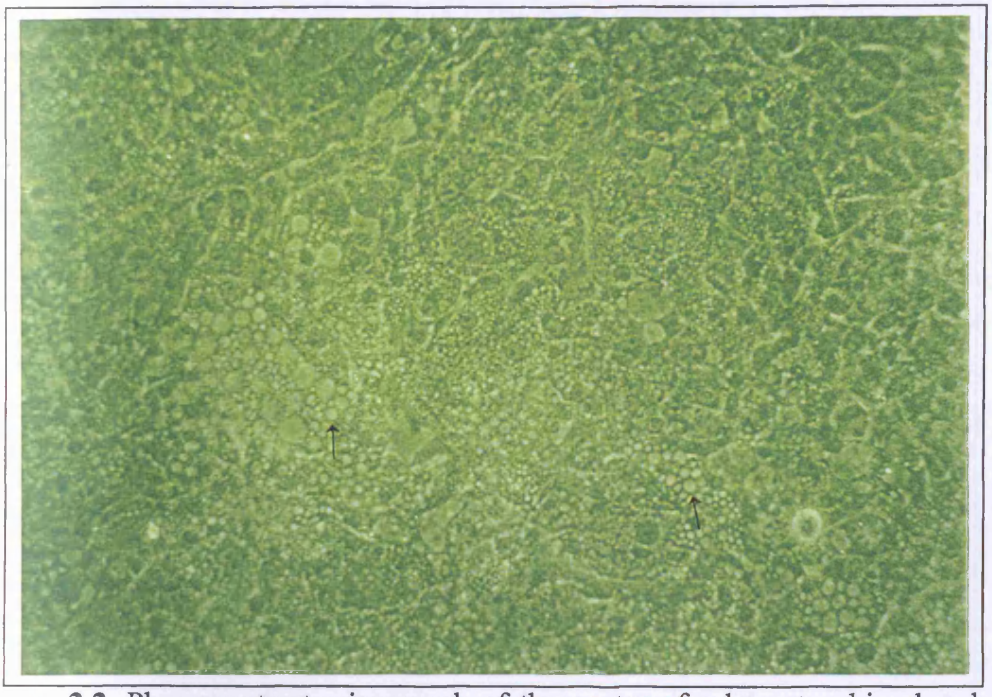


**Figure 3.2.** Phase contrast micrograph of the centre of a hypertrophic chondrocyte micromass surrounded by a confluent monolayer of BAEC. There has been a loss of the rounded morphology and the cells are only a monolayer instead of a 3-dimensional mound. Virtually all of the cells contain vacuoles (arrows show typical example), appear degenerate and most have lost their refractive ECM. (x 100).

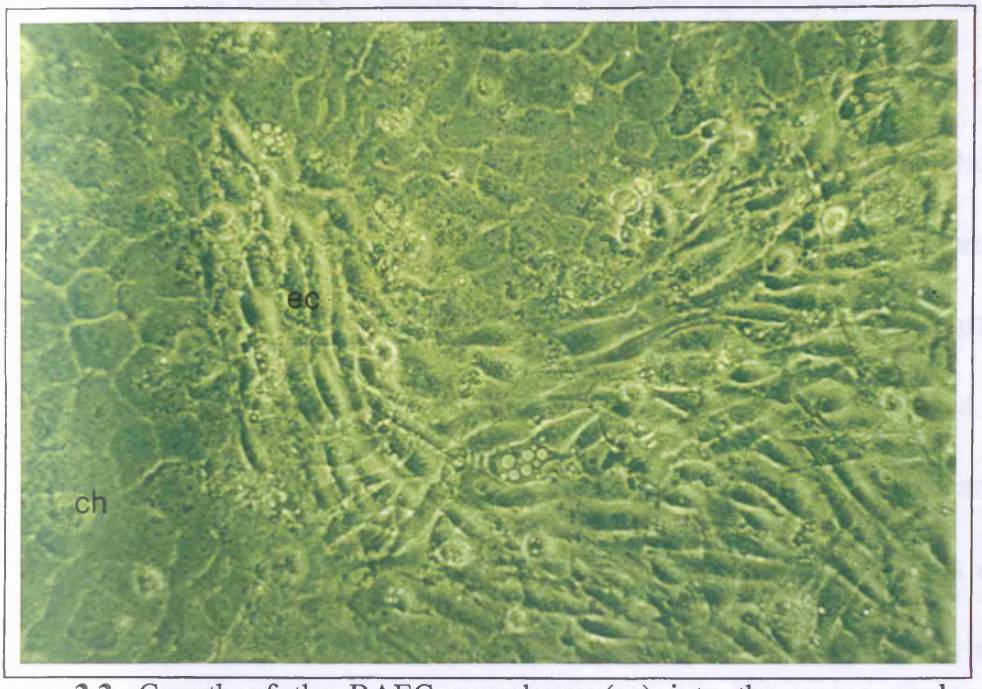



**Figure 3.3.** Growth of the BAEC monolayer (ec) into the now monolayer of hypertrophic chondrocytes. There is a lack of refractive ECM and a few chondrocytes next to the BAEC show the presence of large vacuoles. (x 100).

ii) Hypertrophic chondrocyte micromasses with BAEC micromasses:

BAEC micromasses settled as confluent monolayers on the dish surface. After two days of culture, chondrocytes plated with 3 or 4 BAEC micomasses had lost most of their three dimensionality and had become granular, a number of the cells also contained large vacuoles (see Figure 3.2), those with one or two BAEC micromasses appeared normal. By the fourth day of culture, chondrocytes plated with 3 or 4 BAEC micromasses showed much degeneration, the colony had completely lost its three dimensionality and the cells were very granular, containing many large vacuoles. There was also a loss of the refractive ECM compared with control cultures of chondrocytes. Chondrocyte micromasses plated with 2 BAEC micromasses showed a loss of three dimensionality and refractive ECM with some slight granularity of the cells. Those plated with only 1 BAEC micromass still appeared normal.

After 6 days, chondrocytes plated with 3 or 4 BAEC micromasses were extremely degenerate and showed the beginnings of cell death, indicated by a loss of cells and the appearance of floating debris (Figure 3.4). Those chondrocytes with 2 BAEC micromasses were also very degenerate, and those with only 1 micromass were beginning to show signs of degeneration via some granulation and vacuolation (see Figure 3.2).

After 7 days, chondrocytes plated with 2, 3 or 4 BAEC micromasses were either totally degenerate or almost completely absent, seen by much cell

debris and very few cells left intact on the dish surface, and by 8-9 days the chondrocytes plated with 1 BAEC micromass were also in a similar state. Throughout the entire culture period, the endothelial cells had never proliferated enough to come into contact with the chondrocyte micromass suggesting that a soluble factor may be involved. Control chondrocytes remained healthy throughout the culture period.

iii) Hypertrophic chondrocyte micromasses with ECCM:

The effect of the ECCM on the chondrocyte micromasses varied slightly in the timing of its effect each time the experiment was performed.

*100%:* Micromasses treated with undiluted ECCM began to lose their refractive ECM and their three dimensionality, while becoming granular and vacuolar, by 2 days (see Figure 3.2). The edges of the colonies became fibroblastic (Figure 3.5), and by 7-8 days of culture the cells were very degenerate with much floating debris. By 10 days, one of two things happened: i) Generally the colonies were completely degenerate, with perhaps a few fibroblastic cells remaining. ii) In occasional cultures, the cells were seen to carry on proliferating and became polygonal again, forming a complete monolayer, some colonies of cells having a refractive ECM. The timing of this varied from 15 days up to 20 days. Once confluent the cells would rapidly degenerate and disappear within 48 hours.

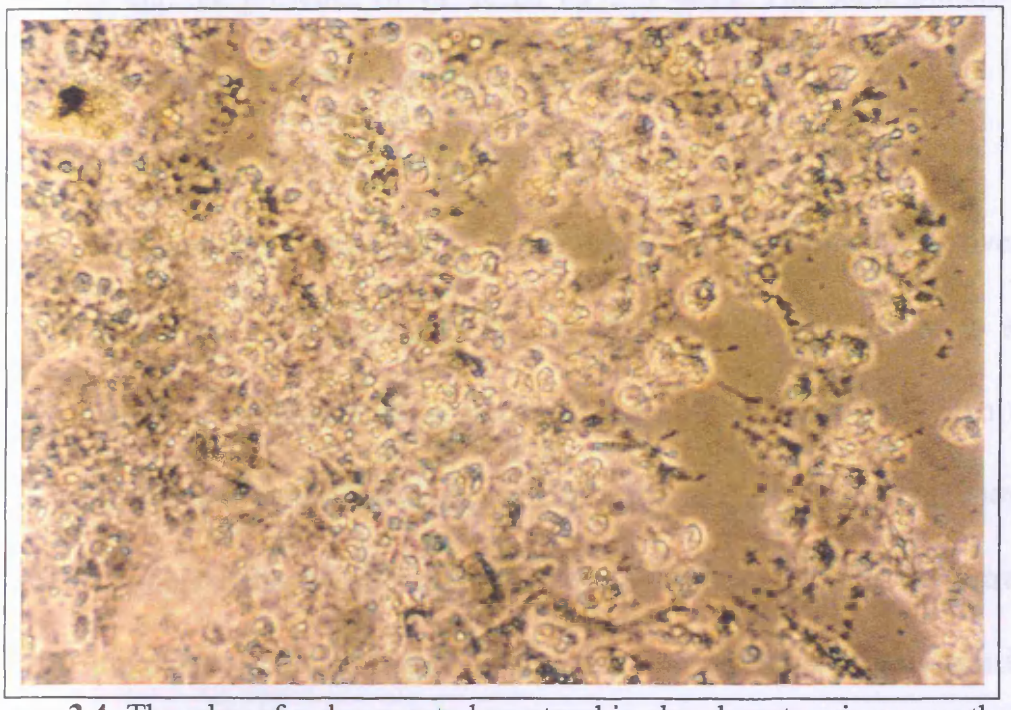


**Figure 3.4.** The edge of a degenerate hypertrophic chondrocyte micromass that had been plated with 4 BAEC micromasses. A number of cells have floated off and there is a large amount of floating debris. (Phase contrast, x 100).

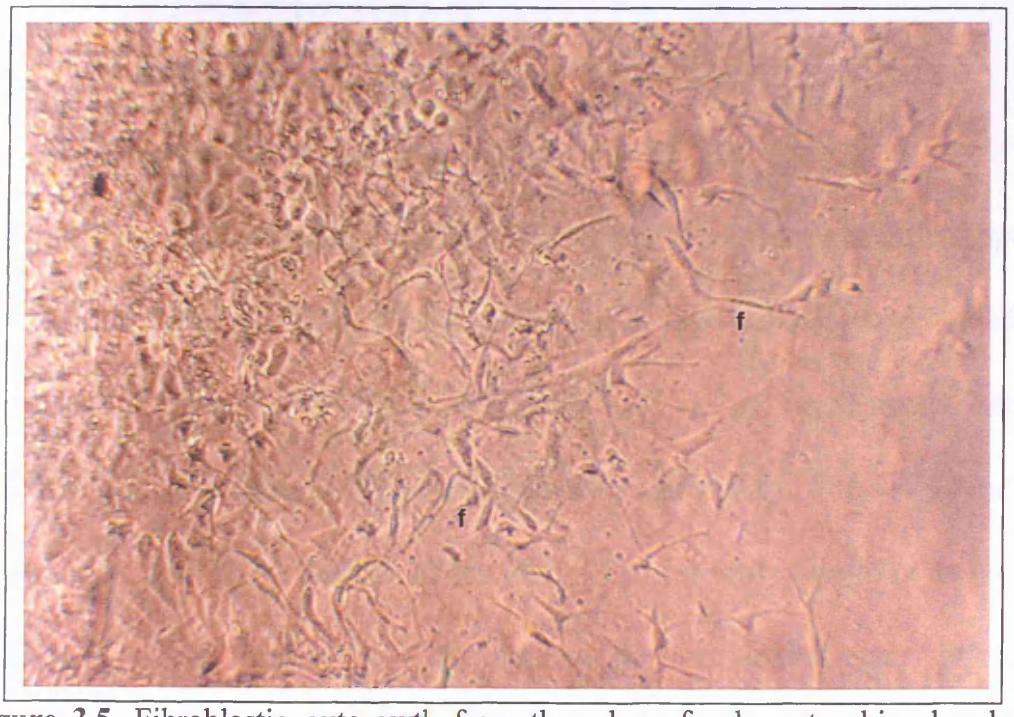



**Figure 3.5.** Fibroblastic outgrowth from the edge of a hypertrophic chondrocyte micromass treated with ECCM (typical examples indicated by f's). (Phase contrast, x 100).

*80%:* These cultures showed loss of ECM and 3-dimensionality, along with granularity and the presence of vacuoles by 3-4 days. The same pattern was then followed as for the 100% treated cells.

*20-60%:* These cultures remained morphologically similar to controls over the first 5-6 days. By 7-8 days the 40% and 60% cultures did begin to show similar symptoms of degeneration as described above. These cells then went on to behave in an identical fashion to the 100% and 80% cultures, showing cell death at approximately the same total time as these cultures. 20% treated cultures remained virtually healthy throughout the culture period, though some fibroblastic outgrowth and some granularity in excess of control cultures was eventually seen.

iv) Hypertrophic chondrocyte monolayers with ECCM:

Chondrocytes settled rapidly, and after being left overnight generally appeared as rounded cells and were very refractive, a few cells having settled and appeared polygonal with some refractive ECM, as is normally seen in hypertrophic chondrocytes (Rooney, 1984) (Figure 3.6). The cells tended to accumulate near to the border of the dish, leaving only single cells or small groups of two or three cells in the central portion of the dish. Conditioned medium was added at this point. Only conditioned medium from confluent

endothelial cells caused the effects described below, conditioned medium from sub-confluent endothelial cells had no visible effect on the chondrocytes.

As with the treatment of micromasses with ECCM, differences were again seen in the time scale of the effects and the ultimate death of the cells. A general scheme is described.

*0-2 Days Treatment:-*

Most control chondrocytes remained rounded and refractive, while a number became polygonal with some refractive ECM. The majority of ECCM treated chondrocytes had settled onto the plastic surface, mainly as small colonies of polygonal cells with a refractive ECM, though a small number of the cells had become fibroblastic (Figure 3.7). A number of the polygonal cells contained small vacuoles.

*2-5 Days:-*

Over this period, most control chondrocytes settled from their round phenotype, onto the plastic as polygonal cells with a refractive ECM, mainly as small colonies, often with a few fibroblastic cells growing in amongst them. Some of the cells were still rounded. The cells appeared healthy. There was often a number of floating cells which, when removed, pelleted and plated out, settled as normal hypertrophic chondrocytes.

A number of ECCM treated cells appeared to have shrunk and become very small and rounded, the membrane appearing slightly crenated. Under the phase contrast microscope, some of these cells appeared shiny, others appeared dark and granular. Most of the remaining cells appeared as either polygonal cells, fibroblastic cells or had many extended processes, almost star shaped. The polygonal cells contained vacuoles, and were granular, occasionally with some slightly refractive ECM. The fibroblastic cells on the whole appeared healthy and did not contain vacuoles. In some cases there were large fibroblastic colonies, and often areas of possible necrosis, shown by cell debris (Figure 3.8).

*5-7 days:-*

Control cells were generally in large colonies of polygonal cells producing a cobblestone pattern with much refractive ECM, there was also a number of fibroblastic colonies often dispersed within the polygonal cell colonies. Large numbers of floating cells were still observed, and if removed and plated out, settled as normal hypertrophic chondrocytes.

ECCM treated cells appeared largely degenerate. There was a lot of floating debris and the attached cells were either condensed with crenated cell membranes or were fibroblastic. There was also the occasional vacuolated polygonal cell (Figure 3.9).

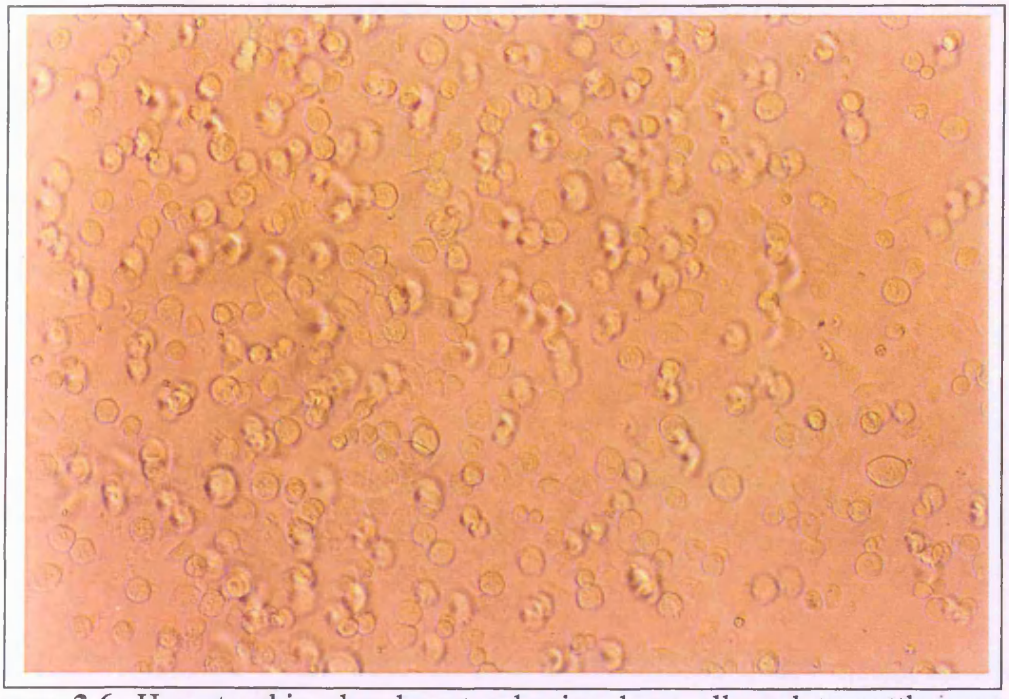


**Figure 3.6.** Hypertrophic chondrocytes having been allowed to settle overnight. Many of the cells still maintain a rounded configuration. (Phase contrast, x 100).

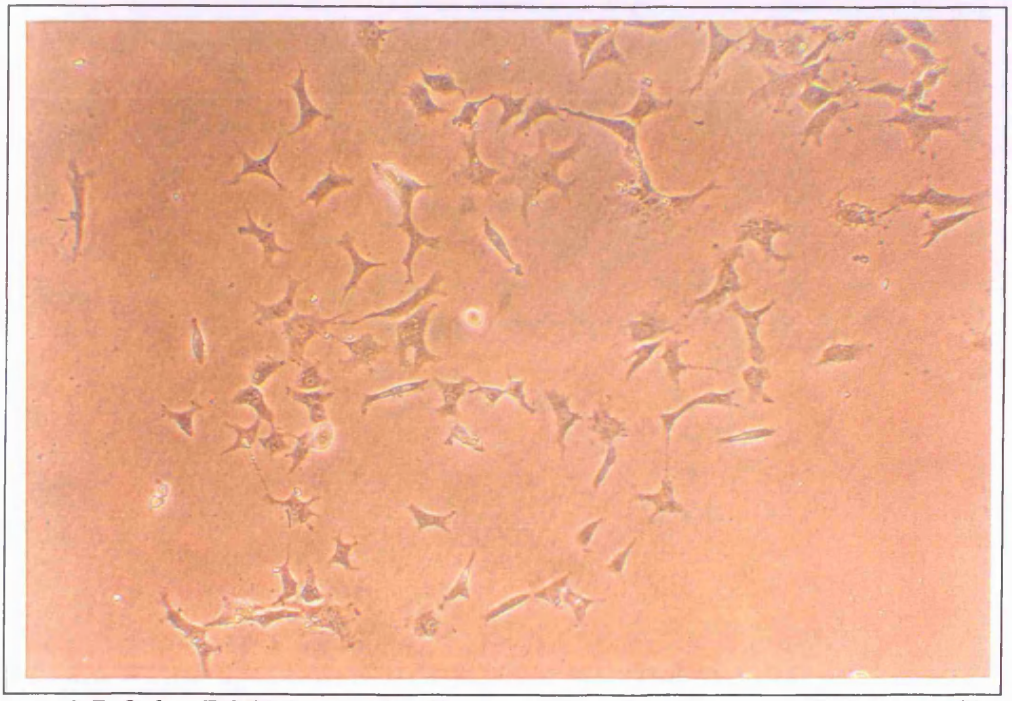


**Figure 3.7.** 2 day ECCM treated cells showing a change in morphology to fibroblast like cells. (Phase contrast, x 100).

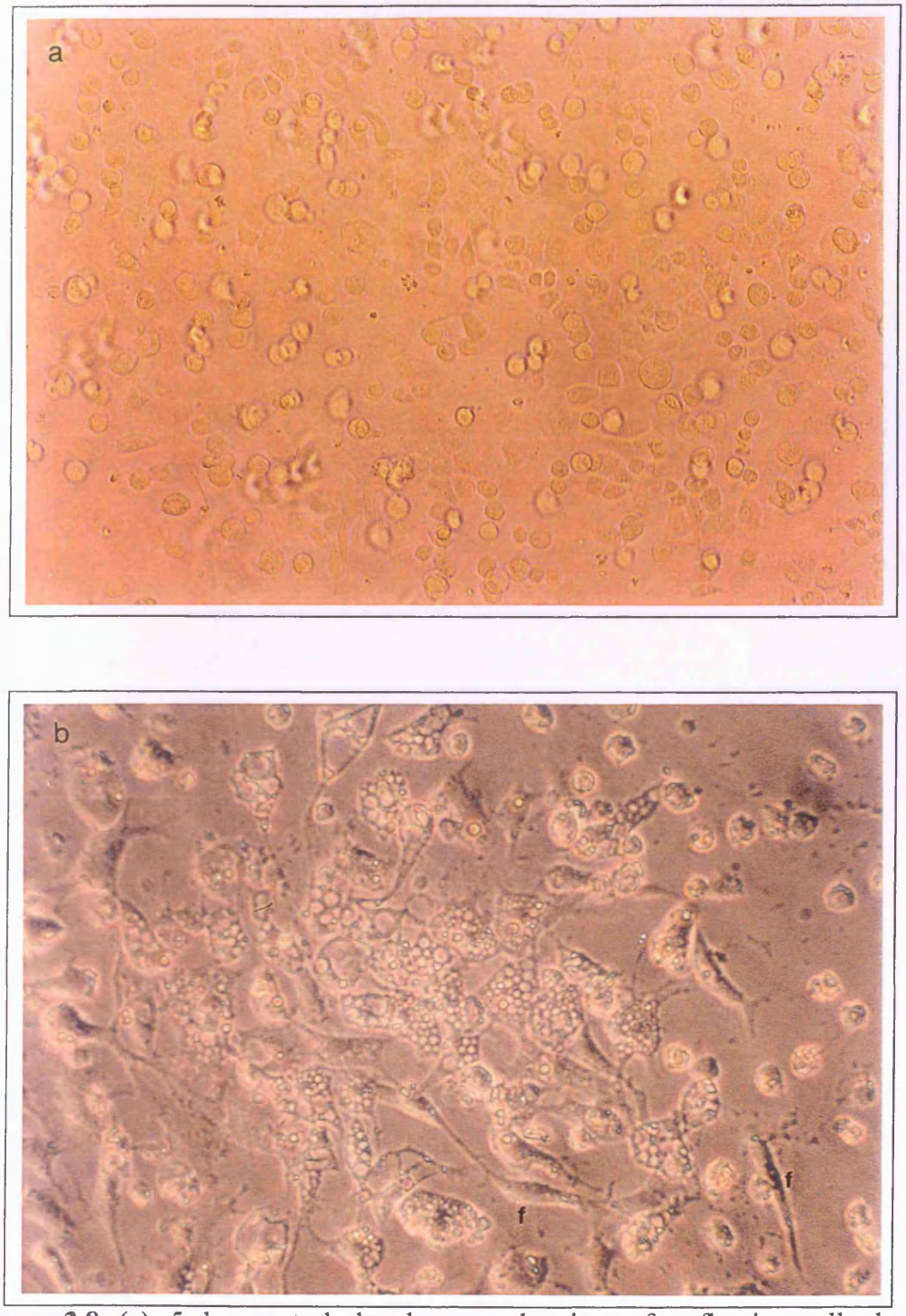



**Figure 3.8. (a).** 5 day control chondrocytes showing a few floating cells, but the majority are settled polygonal cells with refractive ECM. (Phase contrast, x 100). **(b).** 5 day ECCM treated chondrocytes. Most of the cells contain large vacuoles, in amongst which are several fibroblasts (f). (Phase contrast, x 200).

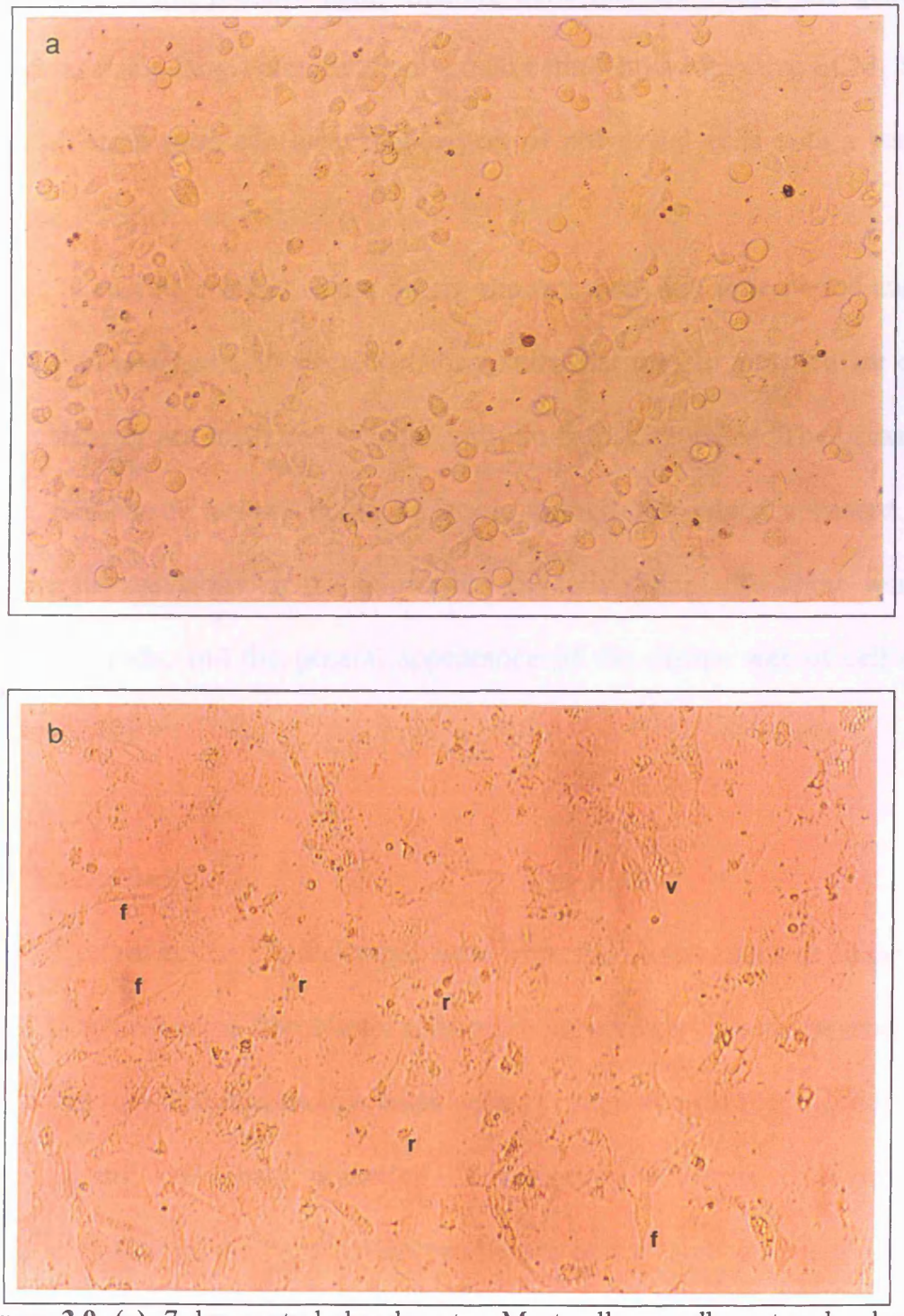



**Figure 3.9. (a).** 7 day control chondrocytes. Most cells are adherent and polygonal with a refractive ECM. Some floating cells are still present. (Phase contrast x 100). **(b).** 7 day ECCM treated chondrocytes. Adherent cells are either fibroblastic (f) or shrunken and rounded up (r) and there is the occassional vacuolated cell (v). (Phase contrast, x 100).

*7-10 days:*

Control cells still produce many floating cells that are viable and generally appear as many large colonies of polygonal cells with a refractive ECM. These cells go on to form confluent monolayers of polygonal cells with a variable lifespan.

In ECCM cultures, more debris appears over this time period and the number of attached cells decreases. Any cells that remain attached are either fibroblastic or are small and rounded with no refractive ECM. The occasional large fibroblastic colony could be seen, though the edges appeared very degenerate, again giving the impression of cells dying off. There were no polygonal cells, and the general appearance of the dishes was of cell death (Figure 3.10).

*12 days onwards:*

Control cultures had changed little from the observations at 10 days. In ECCM cultures some fibroblastic cells often survive the general degeneration and go on to proliferate, though much debris is seen at each feeding indicating there is still cell death occurring. Investigation of these cells was not performed, though the cells do not proliferate at any great rate as the dishes remain mostly bare of cells on subsequent viewings.

The ambiguity of the effects occurred in at least two manners. Firstly, the cells behaved similarly over the first 5 days, but instead of continuing to degenerate, by 7 days, occasional cultures seemed to recover and proliferate to form an almost confluent monolayer of polygonal or cobblestone-like cells with little refractive ECM over the following 5-7 days. However, amongst these cells lay many colonies of various sizes showing a more rounded phenotype and containing much refractive ECM, suggesting some sort of recovery to a chondrocytic phenotype These dishes of cells continued to survive for anything up to 25 days before undergoing rapid cell death. These cells were not investigated further.

Secondly, the cells occasionally had died off almost completely after 5-6 days, behaving similarly to the scheme described above for the first 2-3 days, then very rapidly degenerating.

Cultures treated with ECCM that had been frozen at -20°C for up to 6 weeks, behaved in identical ways to the schemes described above. The ECCM therefore remains stable at sub-zero temperatures for short periods of time.

Removal of conditioned medium at various time points indicated that cells exposed to the medium for at least 12 hours changed morphology, degenerated and died in the same manner as described above, despite replacing the ECCM with control medium, indicating that exposure to the ECCM for as little as 12

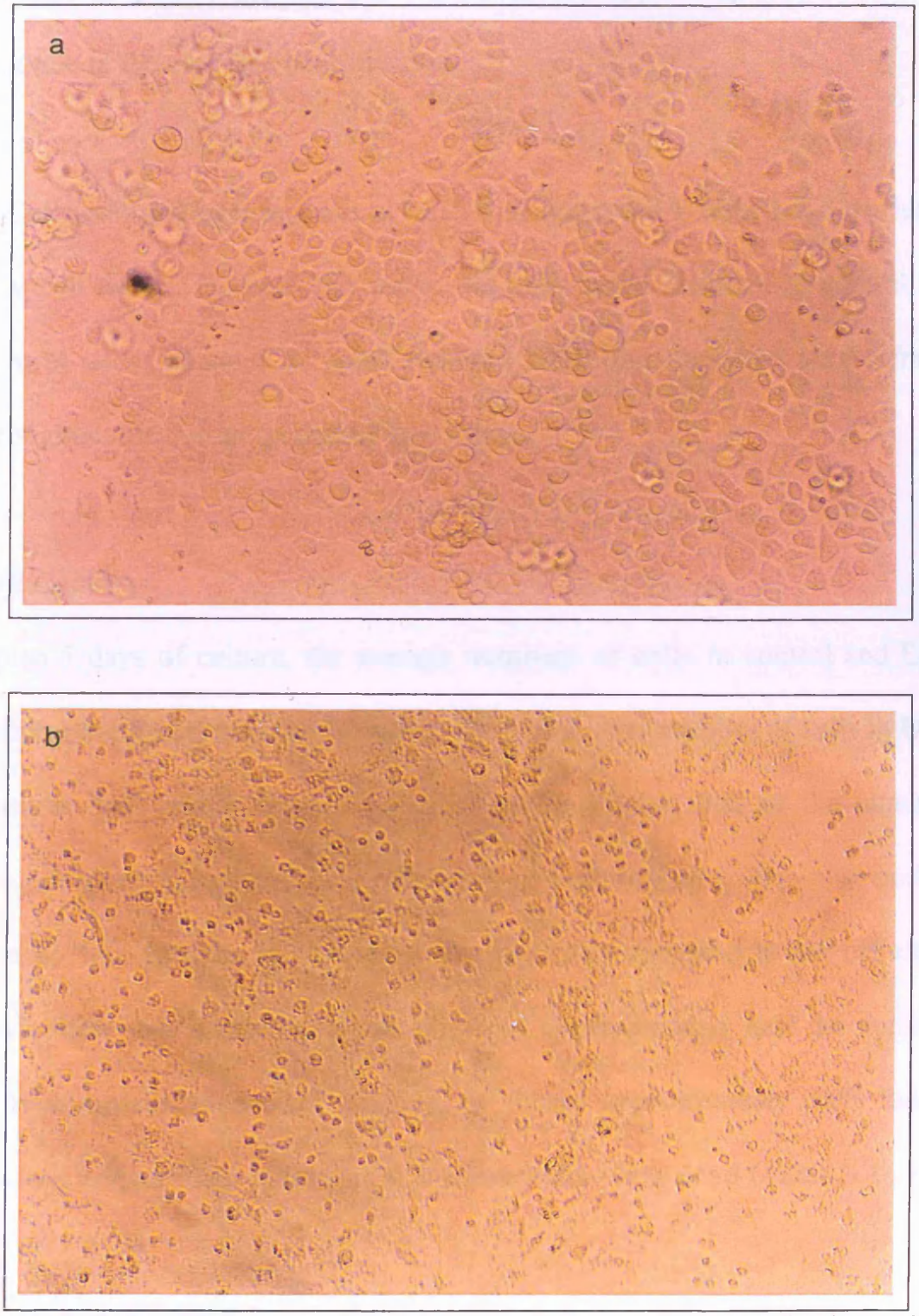



**Figure 3.10. (a).** 10 day control chondrocytes. Cells are adherent and polygonal with much refractive ECM. (Phase contrast, x 100). **(b).** 10 day ECCM treated chondrocytes. Virtually all cells are shrunken and rounded up, though are still adherent. There are no polygonal cells, though occasional fibroblasts were seen. (Phase contrast, x 100).

hours is sufficient to trigger degeneration and death of the cells. Those cultured in ECCM for less than 12 hours are apparently not effected, and continue to develop like control cultures.

v) Cells cultured over agarose in ECCM disappeared within 2-3 days, leaving only cell debris. Up until this point, the cells were identical to control cells. Control cells remained as small rounded cells and appeared very refractive throughout the culture period of 10-12 days.

*Cell Counts:*

Up to 5 days of culture, the average numbers of cells in control and ECCM cultures were very similar. However, by 7 days, the number of cells in ECCM cultures had greatly increased to just under double that of the number in control cultures. In all of these cultures, cell viability via trypan blue exclusion was 85% or greater. In cultures where the cells appeared to die off after 10 days, there was a rapid decrease down to approximately half the number of cells compared to control cultures, of these, approximately 50% failed to exclude trypan blue, indicating that these cells were dead (Table 3.1., Figure 3.11)

**Table 3.1.** Table of cell numbers of control and ECCM treated hypertrophic chondrocytes with the respective cell viability.

| No. of Days | Average no. Control cells | % Viability | Average no. ECCM treated cells | % Viability |
|---|---|---|---|---|
| 1 | $8.18x10^4$ | >95 | $9.4x10^4$ | >95 |
| 2 | $1.24x10^5$ | >95 | $1.14x10^5$ | >95 |
| 5 | $1.74x10^5$ | >95 | $1.63x10^5$ | >90 |
| 7 | $2.3x10^5$ | >90 | $4.7x10^5$ | 85 |
| 10 | $2.56x10^5$ | >90 | $1.47x10^5$ | 52 |

*Alcian Blue Binding Assay*

Using spectrophotometric analysis, ECCM treated cultures contained approximately 30% of the proteoglycan of controls at 2 days of treatment. By 10 days, treated cells contain less than 5% of control proteoglycan levels (Table 3.2., Figure 3.12)

**Table 3.2.** Mean Alcian Blue Binding of Control and ECCM Cultures

| Day | Control absorbance | ECCM absorbance | ECCM as % of Control |
|---|---|---|---|
| 1 | 0.0592 | 0.0468 | 79.1 |
| 2 | 0.0656 | 0.024 | 36.6 |
| 5 | 0.0775 | 0.0153 | 19.78 |
| 7 | 0.1373 | 0.0183 | 13.35 |
| 10 | 0.398 | 0.0143 | 3.6 |

*BrdU Labelling:*

Positive cells were identified by darkly stained brown nuclei (Figure 3.13). By 2 days, 6 times as many ECCM treated cells had incorporated BrdU compared to control cells. This trend was similar at both 5 and 7 days, but by 10 days the number of incorporating cells had declined to below the level of control cultures (Table 3.3., Figure 3.14).

**Table 3.3.** Mean Percentages of BrdU Labelled Cells

| Day | Control % | ECCM % |
|---|---|---|
| 1 | 1.9 | 2.26 |
| 2 | 1.70 | 8.00 |
| 5 | 3.96 | 16.30 |
| 7 | 3.50 | 7.80 |
| 10 | 4.33 | 1.97 |

*Culture of Other Cells in Conditioned Medium*

To test whether the effect of the ECCM on hypertrophic chondrocytes was specific, various other cell types were cultured in conditioned medium.

Chick embryo rounded chondrocytes showed a very rapid initial increase in cell number in the first 2 days compared to control cells, then declined to approximately one third the number of control cells by day 10, though a high proportion of these cells were trypan blue positive. Control cells increased in number over the first seven days, the numbers then slightly

decreased by day 10 (Table 3.4. Figure 3.15).

**Table 3.4.** Table of cell numbers and respective viability in control and ECCM treated 10 day chick embryo rounded chondrocytes.

| Day | Control No. | % viability | ECCM No. | % viability |
|---|---|---|---|---|
| 1 | $1.87x10^5$ | >95 | $2.86x10^5$ | >95 |
| 2 | $2.25x10^5$ | >95 | $4.3x10^5$ | >95 |
| 5 | $4.87x10^5$ | >95 | $2.7x10^5$ | >95 |
| 7 | $5.9x10^5$ | >95 | $2.5x10^5$ | 29 |
| 10 | $5.6x10^5$ | >85 | $1.7x10^5$ | 61 |

Human OA chondrocytes, in terms of proliferation and cell death followed a similar pattern to the hypertrophic chondrocytes, except that throughout the culture period, there were no signs of cell degeneration in the early culture stages, the chondrocytes (in de-differentiated state) contained no vacuoles, though the eventual degeneration, seen at approximately 10 days of culture, was similar to that of the hypertrophic chondrocytes in that the cells shrunk, rounded up and many floated off. A high proportion of the cells at this stage could not exclude trypan blue (Table 3.5., Figure 3.15).

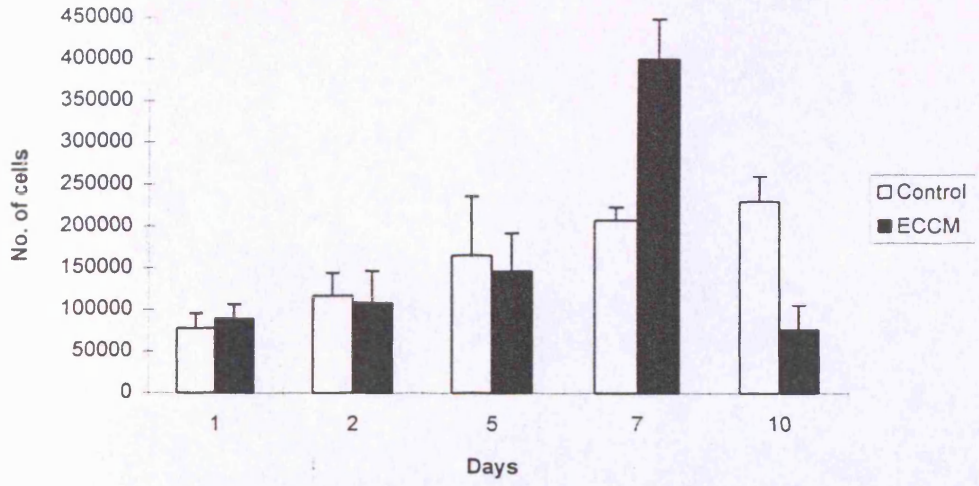



**Figure 3.11.** Comparison of viable cell counts of control and conditioned medium treated hypertrophic chondrocytes. Counts at 7 and 10 days were statistically significant ($p<0.005$) (Bars = 2SD).

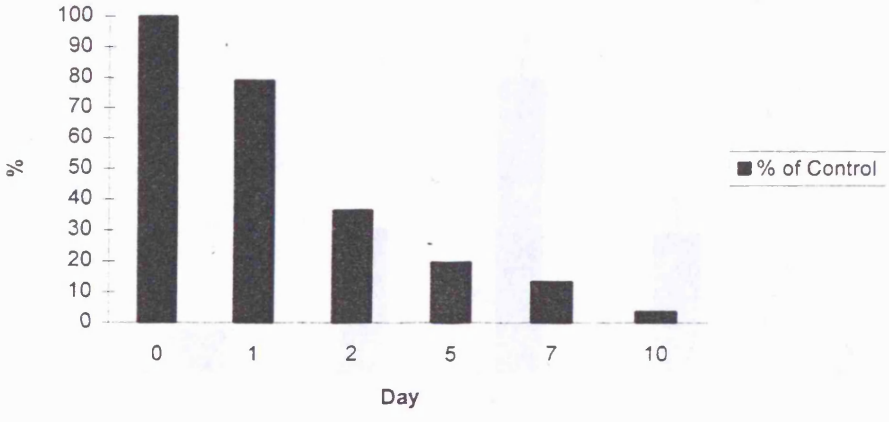



**Figure 3.12.** Percentage of alcian blue binding of conditioned medium cultures compared to control cultures.

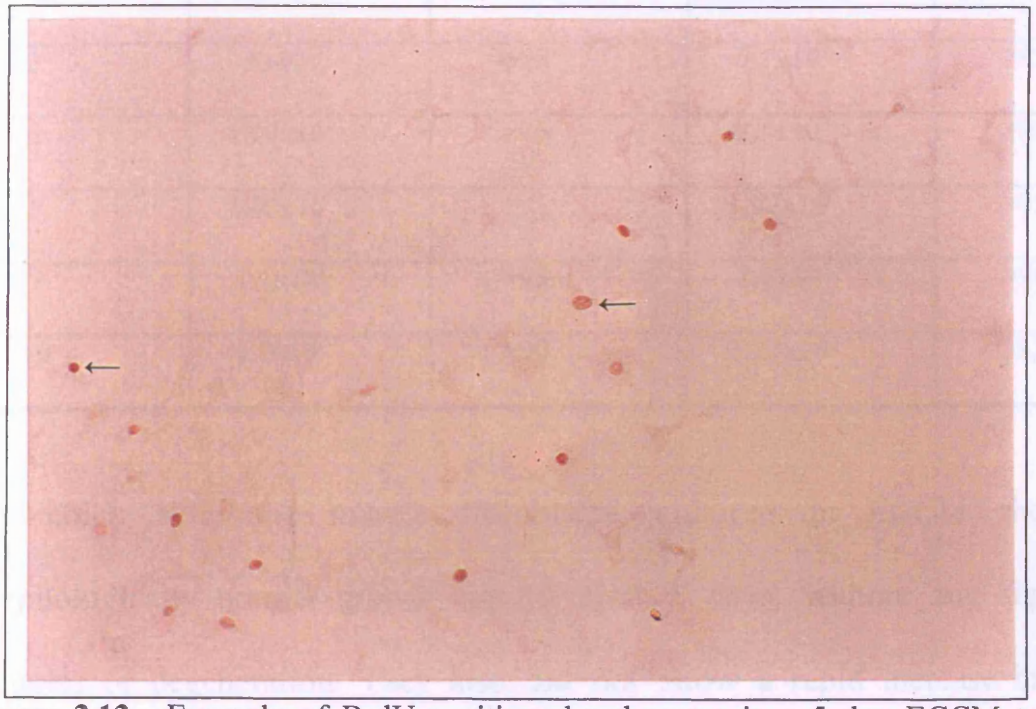


**Figure 3.13.** Example of BrdU positive chondrocytes in a 5 day ECCM treated culture (arrows indicate typical positive cells). (x 100).

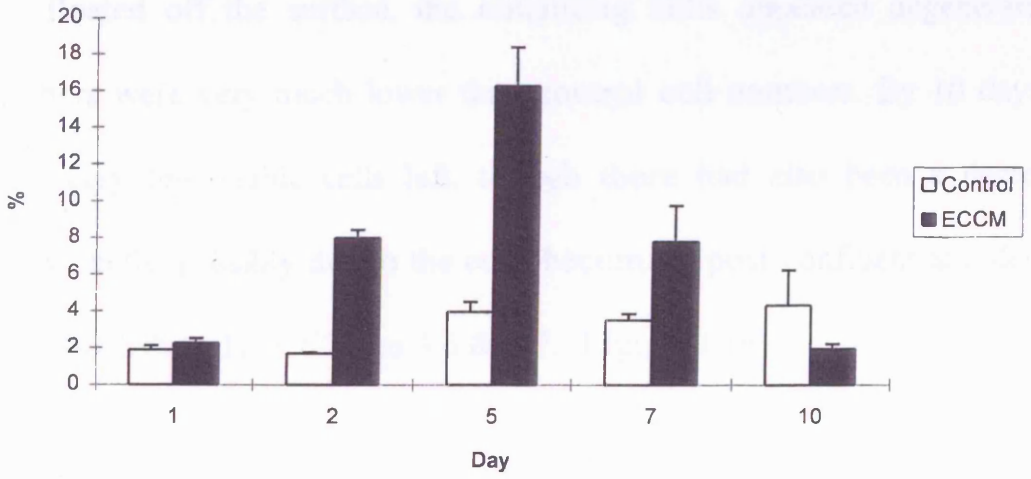



**Figure 3.14.** Comparison of the percentage of BrdU positive cells in control and ECCM cultures. (Bars = 2SD)

**Table 3.5.** Table of cell numbers and respective viability in control and ECCM treated human OA chondrocytes.

| Day | Control No. | % viability | ECCM No. | % viability |
|---|---|---|---|---|
| 1 | $1x10^5$ | >95 | $9.1x10^4$ | >95 |
| 2 | $1.09x10^5$ | >95 | $8.5x10^4$ | >95 |
| 5 | $1.05x10^5$ | >95 | $1.57x10^5$ | >95 |
| 7 | $1.1x10^5$ | >95 | $1.6x10^5$ | >90 |
| 10 | $1.2x10^5$ | >90 | $3.25x10^4$ | 53.8 |

The chick skin and muscle fibroblasts cultured in ECCM showed morphologically normal growth for the first 5 days, without any sign of vacuoles or degeneration. They also did not show a rapid increase in cell number over the first 7 days as seen with the chondrocytes. The cell numbers in fact, did not vary much compared to controls. By 7 days, many of the cells had floated off the surface, the remaining cells appeared degenerate. Cell numbers were very much lower than control cell numbers. By 10 days there were very few viable cells left, though there had also been a decrease in control cells, possibly due to the cells becoming post confluent and detaching from the dish surface. (Tables 3.6 & 3.7., Figure 3.16).

**Table 3.6.** Table of cell numbers and respective viability of chick skin fibroblasts in control and ECCM cultures.

| Day | Control No. | % viability | ECCM No. | % viability |
|---|---|---|---|---|
| 1 | $1.19x10^5$ | >95 | $9.02x10^4$ | >95 |
| 2 | $1.28x10^5$ | >95 | $9.62x10^4$ | >95 |
| 5 | $1.87x10^5$ | >90 | $1.31x10^5$ | >95 |
| 7 | $2.98x10^5$ | >90 | $5x10^4$ | 42 |
| 10 | $1.07x10^5$ | >80 | $3x10^4$ | 46 |

**Table 3.7.** Table of cell numbers and respective viability of chick muscle fibroblasts in control and ECCM cultures.

| Day | Control No. | % viability | ECCM No. | % viability |
|---|---|---|---|---|
| 1 | $1.35x10^5$ | >95 | $1.32x10^5$ | >95 |
| 2 | $1.6x10^5$ | >95 | $1.29x10^5$ | >95 |
| 5 | $1.48x10^5$ | >90 | $1.28x10^5$ | >95 |
| 7 | $2.29x10^5$ | >90 | $4.37x10^4$ | 62 |
| 10 | $1.27x10^5$ | >85 | $1.75x10^4$ | 55 |

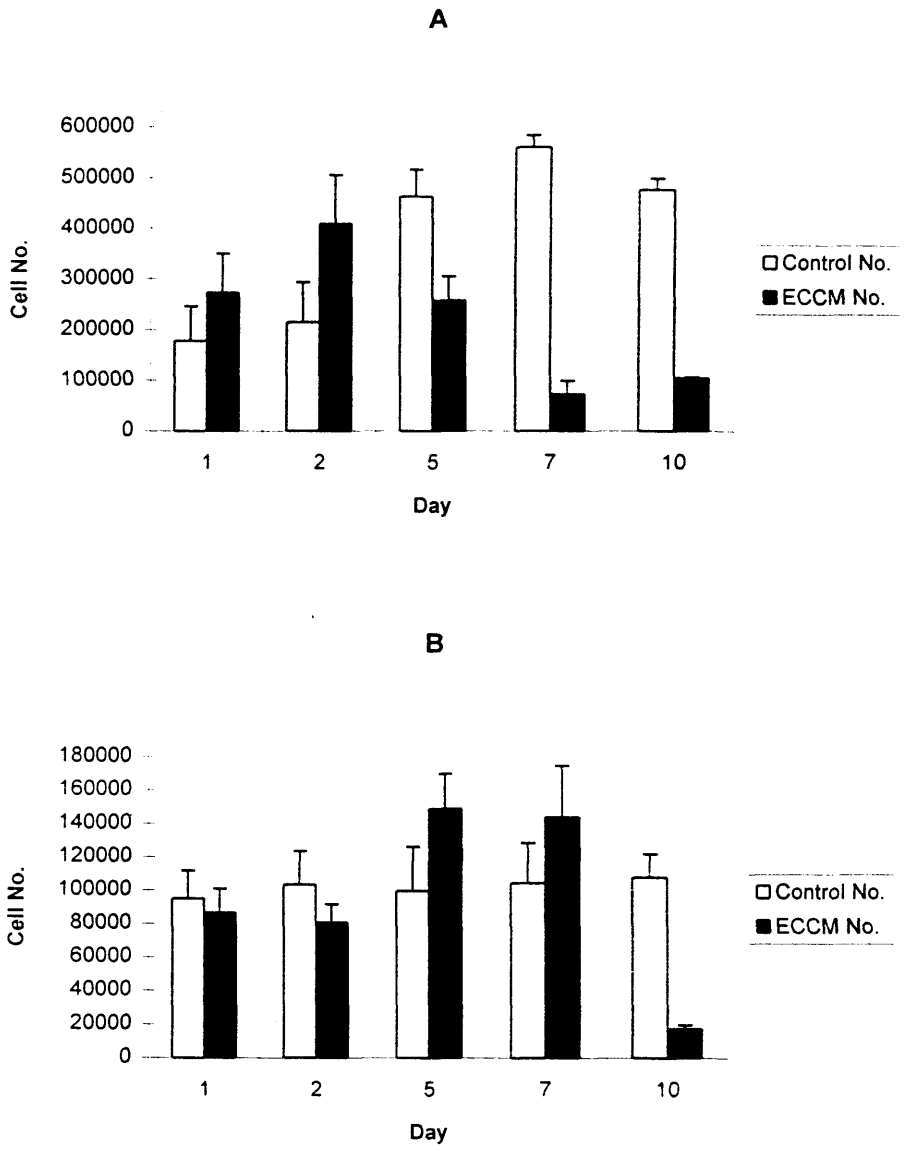



**Figure 3.15. A.** Comparison of control and conditioned medium treated viable chick rounded chondrocyte numbers. All counts were statistically significant ($p<0.005$) **B.** Comparison of control and conditioned medium treated viable human osteoarthritic articular cartilage chondrocytes. All counts, except those at day 1, were statistically significant (($p<0.005$) (Bars = 2SD).

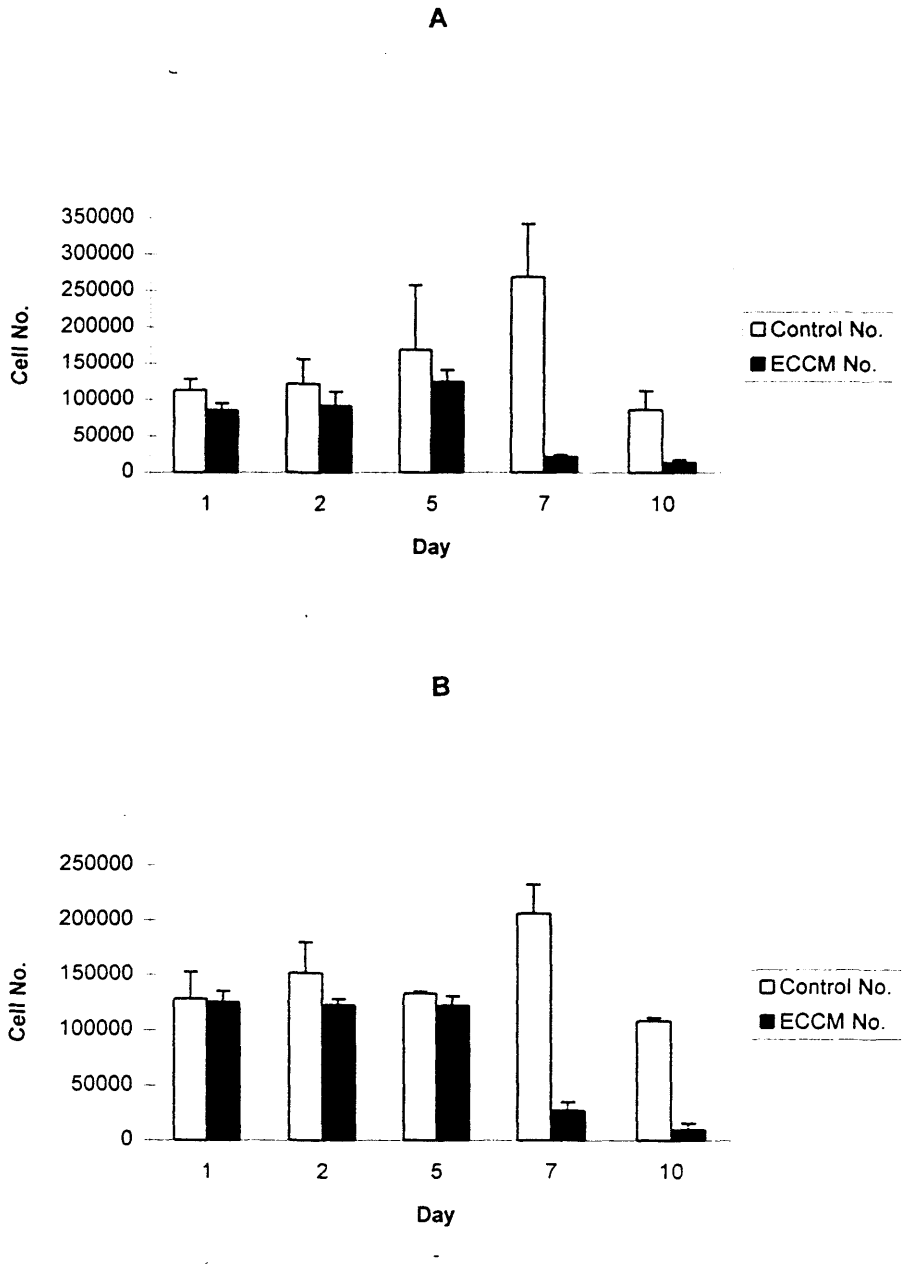



**Figure 3.16.** Comparison of control and conditioned medium treated chick fibroblast numbers from **A.** skin (All counts were statistically significant, $p<0.005$) and **B.** muscle (All counts, except day 1, were statistically significant, $p<0.005$). (Bars = 2SD)

## 3.4. DISCUSSION

Described here is an interaction, *in-vitro*, between chick embryo hypertrophic chondrocytes and BAEC. Although the cells are derived from different species, a similar system has successfully been used previously to study the interaction of chick chondrocytes and bovine aortic rings (Pepper *et al.*, 1991). Interestingly, the work presented in this chapter provides results that are completely opposite to those achieved by Pepper *et al.*, (1991), in that the work by these authors showed an inhibition of endothelial growth caused by the chondrocytes. However, these authors used chick embryonic sternal chondrocytes as opposed to hypertrophic chondrocytes and bovine microvascular endothelial cells instead of BAEC. The work here showed an adverse effect of the endothelium on the chondrocytes.

The results presented here indicate that the vasculature plays a major role in the degeneration and subsequent removal of hypertrophic cartilage during development which supports the interpretation of Gibson *et al.*, (1995b)

The presence of a confluent layer of endothelial cells around a hypertrophic chondrocyte micromass leads to a rapid loss of refractive matrix, degeneration and finally removal, of the chondrocytes, perhaps mimicking the removal of hypertrophic cartilage during the development of long bones. This would seem to be an active process due to the initial degeneration of the chondrocytes as opposed to the endothelial cells simply overgrowing or

pushing the chondrocytes off the surface. However, the physical presence of the endothelial cells is not a pre-requisite for chondrocyte degeneration. A soluble factor or factors released into the growth medium by a confluent layer of endothelial cells is enough to trigger the same sequence of events in these hypertrophic chondrocytes. This provides evidence for a vascular based trigger of cartilage degeneration and subsequent vascular removal of cartilage. The need for the endothelial cells to be confluent and therefore in direct cell-cell contact, is most likely physiological in that endothelial cells are unlikely to exist as single entities within the body. However, it may simply be that sub-confluent endothelial cells do not produce a high enough concentration of the factor(s) due to lack of cell numbers.

The ambiguity in the effect of the confluent ECCM soluble factor(s) on the chondrocytes may simply be explained by a lack of quantitation. The effects of diluting the conditioned medium, and culturing with different numbers of BAEC micromasses, showed that the effects are concentration dependent. The amount of medium added at each feed, though equal in volume throughout the culture period, is unlikely to contain an equal amount of active factors as there is no guarantee that the endothelial cells have produced identical amounts of factor(s) each time that fresh ECCM is generated. For instance, more factor(s) may be produced when the cells have just reached confluency, the amount decreasing during the time that the cells are

maintained in a confluent state, or vice versa. If this is the case, then at each feed, the chondrocytes are exposed to varying levels of factor(s), which may be responsible for causing the variation in the effect. Also, the passage number at which the endothelial cells are at may also affect the production of the factor(s). To determine the effect of time of culture, and the effect of the passage number of the BAEC, ECCM could be taken from endothelial cells of particular passages and the feeding of chondrocyte cultures limited to this medium only. Medium could also be extracted at exact times post-confluence i.e. 1 day, 2 days etc. and again, chondrocyte cultures limited to feeding with ECCM from only one particular day. However, despite any possible variations in the factor(s) concentration, the ultimate fate of the chondrocytes is degeneration and death.

There is an indication that the chondrocytes may be able to activate some form of attempted repair mechanism. This is not however a novel concept and has been thought to exist in articular chondrocytes during OA, shown by their increased production of collagen (Lipiello *et al.*, 1977; Ryu *et al.*, 1984), proteoglycans (Collins & McElligott, 1960; Mankin *et al.*, 1971; Ryu *et al.*, 1984) and the formation of chondrones (Archer *et al.*, 1994). Upon initial treatment, the hypertrophic cells lose matrix, become fibroblastic, or remain polygonal and develop vacuoles. With increasing time some cultures were seen to reverse this process and reproduce a more chondrocytic like

phenotype, and refractive ECM for a short while before eventually dying off. These cells were not studied, though may provide further answers as to the metabolism of vascularly invaded cartilage. This process provides some evidence for a repair response.

It is possible that the whole scheme of events could be a repair/survival response. Chondrocytes cultured over agarose, and therefore maintained in a rounded morphology, without cell-surface contacts, disappeared within 2-3 days when cultured with ECCM, also, the hypertrophic chondrocyte micromasses, which allow the cells to remain in the rounded chondrocytic morphology (Ahrens *et al.*, 1977) very rapidly lost their 3-dimensionality to leave only a monolayer, the cells involved in forming the 3-dimensional structure having disappeared. These cells have most likely either died, though there was little indication of floating cells or debris, or, some of them certainly had floated away and settled in other areas of the dish as small colonies and were often visualised at distances from the original micromass. This suggests that the rounded configuration of the cells was more susceptible to the degradatory factor(s) within the conditioned medium. Allowing the cells to settle onto a surface and spread, may provide them with the ability to attempt a survival strategy which involves some form of morphological change. This may be linked to a phenotypical change, as there is a loss of refractive matrix around the cells and a loss of proteoglycan staining as seen by lack of alcian

blue binding after ECCM treatment.

The effect of ECCM factors may not be totally specific to hypertrophic chondrocytes, as ECCM was also shown to have a degenerative effect on other cell types. However, the pattern of degeneration is not the same, and there are several possibilities. First, the factor(s) affecting the other cell types are not the same ones as those affecting the chondrocytes as the ECCM is likely to contain a mixture of molecules, including various cytokines and growth factors. Secondly, the influencing factor(s) may be the same, but act through a different cellular mechanism.

Overall, this work provides evidence for a vascular based trigger of cartilage degeneration during development. One step forward would be to develop the model to the use of human endothelium and human chondrocytes, similar to that used by Tada *et al.*, (1994). However, co-culture on plastic would not suffice, because although chick hypertrophic chondrocytes remain chondrocytic on tissue culture plastic (Rooney, 1984), human chondrocytes do not, and simply dedifferentiate into fibroblasts (Mayne *et al.*, 1976; Kirsch *et al.*, 1992; Bonaventure *et al.*, 1994). Models have developed to maintain chondrocytes in a rounded configuration by the use of gels (Gibson *et al.*, 1982; Castagnola *et al.*, 1988; Archer *et al.*, 1990; Kirsch *et al.*, 1992; Aulthouse, 1994; Gunther *et al.*, 1994) or micromass cultures (Ahrens *et al.*, 1977). In terms of the developing chondrocytes, the models described above

could also be attempted inside gels. The brief work on the effect of the ECCM on hypertrophic cells cultured over agarose should be further investigated, to determine if the rounded morphology is truly more susceptible to the effects of the vasculature.

An explanation for the whole process is that the cells are simply dying slowly. *In-ovo*, these cells would be removed by the active vasculature, and would be unlikely to have time to change phenotype, or even have the need to change phenotype. In the model using ECCM, the cells are not exposed to an active vascular system, instead, being only exposed to factors produced by the vasculature, which even then are not being constantly produced in the culture. The cells cannot be actively removed by other cells. The morphological change may simply be a response to degeneration that never gets the chance to occur *in-ovo*. The cells go through the normal stages of degeneration but are not removed and even get the chance to respond to the degeneration in ways that they would not normally have the chance to do. For instance, the change to a fibroblastic morphology and the increased cell proliferation may be an attempt to survive, or even to repair the damage. *In-ovo* the cells would not get the opportunity to do this before being removed by the vasculature.

# CHAPTER 4

# Collagen Expression of Chick Hypertrophic Chondrocytes Undergoing Endothelial Cell Mediated Degeneration

## 4.1. INTRODUCTION

Cultured monolayers of human articular chondrocytes lose their differentiated phenotype very rapidly to become 'dedifferentiated' cells, and synthesise molecules associated with fibroblastic cells, such as type I collagen (Mayne *et al.*, 1976; Kirsch *et al.*, 1992; Bonaventure *et al.*, 1994). Hypertrophic chondrocytes from the developing cartilage rudiments of chick embryos on the other hand, maintain their normal phenotype and produce normal cartilage molecules (Rooney, 1984). This makes them an ideal model for cell culture studies.

The synthesis of normal cartilage molecules and therefore the chondrocyte phenotype has been associated with the shape of the cell (Archer *et al.*, 1990; Bonaventure *et al.*, 1994). It has been highlighted that the rounded configuration of chondrocytes is vital for the maintenance of collagen type II and cartilage proteoglycan expression, while the acquisition of a fibroblastic (dedifferentiated) morphology is associated with a loss of this cartilage phenotype (see Archer *et al.*, (1990)). However, dedifferentiated chondrocytes have the potential to re-express the cartilage phenotype when cultured under conditions allowing recovery of the rounded morphology (Benya & Shaffer, 1982).

The morphological effect of ECCM medium on chick hypertrophic chondrocytes is discussed in the previous chapter. The first consequence was to cause an apparent dedifferentiation of many of the chondrocytes to a fibroblastic morphology. By using this model an examination of the collagen

expression of ECCM treated chondrocytes has been performed at both the protein and mRNA level, to resolve whether there is a particular change in the expression of cartilage specific collagens to a more widespread collagen species, such as type I collagen.

Examination of mRNA species involved the introduction of reverse transcription - polymerase chain reaction (RT-PCR). This was developed in the laboratory with the aid of Dr. S. Chappell and Dr. Tom Walsh of the Breast Cancer Research Unit (BCRU) at Leicester University. RT-PCR as a technique has been used in several studies to examine collagen expression (Nakata *et al.*, 1992; Dhawan & Beebe, 1994; Fitch *et al.*, 1995; Hopkinson *et al.*, 1995).

The polymerase chain reaction is an enzymatic method of making multiple copies of a segment of DNA. The process of amplification is achieved by the addition of two synthetic oligodeoxynucleotide primers, of specific sequence which bind to opposite strands of the template, a thermostable DNA polymerase, the four deoxyribonucleoside tri-phosphates and a DNA template. This mix is cycled through a program of heating and cooling steps to denature the double stranded DNA template, allow primer annealing to the template and finally synthesis of DNA from the 3' end of the annealed primers (Sambrook *et al.*, 1989; Kocher & Wilson, 1991). PCR can also be used to amplify first-strand cDNA, synthesised by a DNA-dependent RNA polymerase (reverse transcriptase) from a mRNA template. Oligo (dT) is used to prime the reverse transcriptase (RT) reaction (Sambrook *et al.*, 1989).

## 4.2. MATERIALS AND METHODS

*Cells*

Hypertrophic chondrocytes and endothelial cells were extracted as described (Appendix I). Conditioned medium was prepared as previously. Chondrocytes were plated at $1x10^5$ cells per 35mm dish and allowed to settle overnight before treatment with ECCM.

*Radiolabelling of collagens*

24 hours prior to radioactive labelling, cultures were fed with media containing 100μg/ml of β-aminopropionitrile fumarate (β-APN) and 30μg/ml ascorbic acid (both Sigma). After 24 hours of incubation, this media was removed and replaced either with fresh medium or ECCM, each containing β-APN and ascorbic acid as above, plus 10 μCi/ml of $^3$H-proline (Amersham International) and returned to the incubator for a further 24 hours. At the end of this labelling period, labelled media from both control and ECCM treated cultures was removed and the protease inhibitors phenyl-methyl sulphonyl fluoride (PMSF), N-ethyl maleimide (NEM) and EDTA were added to give final concentrations of 2mM, 10mM and 25mM respectively. The samples were frozen at -20°C until ready for use. High molecular weight proteins, including collagens, were precipitated with the equivalent addition of 176mg/ml of ammonium sulphate (approx. 30% saturation) and incubated at 4°C for 24 hours with continual shaking. The precipitate was centrifuged at 13,000rpm for 20 minutes, redissolved in 1ml of 0.5M acetic acid containing 100μg/ml pepsin (Sigma) and

incubated at 4°C for 12-18 hours. The pH of the solution was raised by the addition of four drops of 4M sodium hydroxide to stop the activity of the pepsin (Kirsch *et al.*, 1992). The dissolved precipitate solutions and the remaining supernatants were then extensively dialysed against 0.2M acetic acid at 4°C. The samples were removed from dialysis, 20µl were diluted into 3ml of scintillation fluid (Optiphase HiSafe 3, Wallac) and counted in a Canberra Packard 1500 Tri-Carb liquid scintillation analyser. The equivalent of 20,000cpm of the precipitate solutions were freeze dried and dissolved in loading buffer (Appendix II) before boiling for 3 minutes and running on 8% SDS polyacrylamide gels (SDS-PAGE) in a similar manner to Laemmli, (1970) (Appendix II) in running buffer (Appendix II). If 20,000cpm were not available, the entire sample was freeze dried, dissolved and run. Gels were then fixed for 30 minutes in a fixing solution (Appendix II) before soaking in Amplify scintillant (Amersham International) for a further 30 minutes. The gels were dried and exposed to pre-flashed autoradiography film (Hyperfilm, Amersham International) at -80°C for 10-20 days before development with Kodak Developer and Fixer (Kodak).

The number of radioactive counts per cell and the cpm and percentage incorporation of the radioactive precursor into high molecular weight proteins was calculated from the cell numbers at each time point (see previous chapter).

*Collagen Extraction from Culture Medium for Immunoblotting*

Spent mèdia from both control and ECCM treated cultures at 2, 5, 7 and 10 days were removed and protease inhibitors added as above. The samples were frozen at -20°C until ready for use. High molecular weight proteins were precipitated, redissolved and dialysed as above, before freeze drying the total sample for immunoblotting.

*Immunoblotting*

Immunoblotting was performed by a method adapted from Kirsch *et al.*, (1992). The collagen samples were run on 8% SDS-PAGE as described above, before soaking in transfer buffer (Towbin *et al.*, 1979) (Appendix II) and blotting onto Immobilon-P PVDF transfer membranes (Millipore), via a semi-dry blotter (Semi-Phor, Hoeffer). Once the blots were complete, the membranes were rinsed in PBS twice before blocking in 5% low fat milk (Marvel) in PBS for 1 hour. After rinsing three times in PBS, the blots were probed for either type I, II or III collagen using goat anti-human type I and III collagen antibodies and goat anti-bovine type II collagen antibody (Southern Biotechnologies) at a 1:200 dilution in PBS. Blots were incubated with the respective antibody at room temperature for 1 hour. After three rinses in PBS, the blots were incubated for 1 hour at room temperature with a peroxidase conjugated rabbit anti-goat antibody (Dako) at a 1:2000 dilution in PBS. DAB/$H_2O_2$ solution (Sigma Fast DAB Tablets, Sigma) was used to visualise the collagens.

*Immunocytochemistry*

Cells were fixed at 2, 5, 7 and 10 days of culture in 95% ethanol at -20°C for 20 minutes, then, either stained immediately, or air dried and stored at -20°C until ready for use (Stephens *et al.*, 1992). Dishes were rinsed 3 times in PBS then flooded with a 1:5 dilution (in PBS) of normal rabbit serum (Serotec) for 10 minutes to block non-specific binding. The serum was removed but not rinsed. Primary antibody, goat anti-type I, II or III collagen (as above) were added at a dilution of 1:40 in PBS and incubated at room temperature for 1 hour before removal, rinsing once with PBS then once with TBS. A secondary peroxidase conjugated rabbit anti-goat antibody was added at a 1:200 dilution in TBS and incubated for a further hour. After three rinses in TBS, DAB/$H_2O_2$ solution (Sigma) was added for 5-10 minutes before three further rinses and mounting in glycerine jelly.

***RT-PCR Materials and Methods:***

All components for the technique were performed with, or made up in sterile/autoclaved plasticware or di-ethyl pyrocarbonate (Sigma) treated glassware to prevent RNase contamination.

*mRNA Isolation*

Isolation of mRNA was performed with the Dynabead mRNA isolation kit according to manufacturer's instructions (Dynal). Cells were trypsinised, pelleted at 2,000 rpm and rinsed once in sterile PBS. Cells were then lysed with

lysis/binding buffer and the DNA sheared by forcing the solution 3 times through a 21 gauge needle. This cell lysate was added to pre-prepared oligo dT dynabeads and incubated at room temperature for 5 minutes to allow binding of mRNA. Beads were rinsed with washing buffers before resuspension in 10µl of reverse transcription (RT) buffer (Promega) for use in the subsequent RT-PCR reaction.

*RT-PCR*

RT-PCR was performed according to a method communicated by Dr. S. Chappell (Breast Cancer Research Unit, University of Leicester). 5µl of dynabead suspension were placed into each of two tubes to which 4µl of dNTP mix (Appendix II) and 1µl of RNase inhibitor (Promega) were added. 14.2µl of ultra pure water (Sigma) plus 0.8µl of Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega) was added to one tube. 15µl of ultra pure water (Sigma) was added to the other tube to act as a negative control. Tubes were incubated at 42°C for 1 hour in a Progene thermal cycler (Techne) to allow cDNA synthesis.

*PCR of RT Products*

To amplify RT products, 2.5µl of the RT reaction from the RT+ and RT- reaction were put into separate 0.5ml tubes with 0.5µl of each primer (5 pmol), 6µl of water and 15µl of pre-prepared, pre-optimised PCR buffer (courtesy of Steve Chappell) (Appendix II). Negative controls were set up by replacing the

RT reaction with water. The mix was overlaid with a drop of mineral oil. 0.5µl of a 1 in 5 dilution of Taq DNA polymerase (Gibco) was added as a hot start reaction in a Progene thermal cycler (Techne) during the first 5 min denaturation stage.

The primers used were chosen from published data, and were specific for the chick collagen species being investigated (Table 4.1.). The products were amplified by using the following sequence:

i) 94°C denaturation step for 5 minutes
ii) 35 cycles of
    a) 94°C for 30 seconds for double strand denaturation
    b) specific primer annealing temperature for 30 seconds.
    c) 72°C, to allow cDNA synthesis, for 30 seconds
iii) 7 minute extension step at 72°C.

Specific annealing temperatures for the primers were calculated by adding 5°C to the $T_m$ value of each primer due to a high magnesium content of the PCR buffer (S. Chappell, personal communication), except for G-3-DPH, where the temperature used previously was employed (Gordon *et al*, 1994). If the $T_m$ for the individual primers within a primer set differed, either the average of the two, or the lowest value was used.

*Agarose Gel Electrophoresis*

6µl of 5x loading buffer (Appendix II) was added to PCR products which were run on 2% agarose gels (ICN) containing $5x10^{-5}$ mg/ml ethidium bromide

(ICN) to visualise, alongside PhiX174/Hae III digest DNA markers (Promega) in 1x TAE solution (Appendix II). Visualisation was done on an UV light box in the dark.

**Table 4.1.** Primers used in the PCR reactions.

| mRNA Species | Primer Sequence | Annealing Temp. and Product Length |
|---|---|---|
| G-3-PDH* | sense-GAT CTG CAC TAC ATG GT<br>antisense-GAG CCC ATT GAT CAC A | 45°C<br>101bp |
| α2(I)** | sense-TTA CTC CTC GCG ACT GTA TGC<br>antisense-GCT CAC CAG GAA CAC CTT GAA | 56°C<br>480bp |
| α1(II)** | sense-GCA GAG ACC ATC AAC GGC GGT<br>antisense-CAG GCG CGA GGT CTT CTG CGA | 58°C<br>333bp |
| α1(IX)** | sense-CAG CTG GCA GCC AGT CTT AGG<br>antisense-CTC TGT CCA GCC TGC ATT CGG | 57°C<br>413bp |
| α1(X)** | sense-AAG GGG CCA CCA CAC TTT CTA<br>antisense-TTC TCC AGG CTT CCC TAT CCC | 57°C<br>552bp |

All primers prepared by Genosys

*Gordon *et al*, 1994

**Nakata *et al*, 1992

*PCR Product Sequencing*

To check that the correct mRNA's had been amplified, the PCR products were sequenced using the dRhodamine method (Perkin Elmer Applied Biosystems). First, PCR products were isolated from the agarose. This was performed by excising the band with a clean scalpel. The cDNA was extracted from the agarose via a Gel Extraction Kit according to the manufacturer's instructions (QIAgen), which involved dissolution of the agarose and binding of DNA to a column before eluting with water.

The DNA was sequenced in 8μl ABI Prism dRhodamine Terminator pre-prepared buffer according to manufacturer's instructions (PE Applied Biosystems). 30-90ng of DNA and 3.2pmol of forward primer were added to

the buffer and the reaction made up to 20µl with ultra pure water (Sigma). The reaction was cycled through 25 cycles of 96°C for 30 seconds, then 50°C for 15 seconds followed by 60°C for 4 minutes on a Progene thermal cycler (Techne). The sequenced products were separated from excess dye terminators by addition of the sequence products to a tube containing 50µl of 95% ethanol and 2µl of 3M sodium acetate (Sigma) pH 4.6. The products were precipitated on ice for 10 minutes before pelleting at 13,000rpm for 30 minutes. The pellet was further rinsed in 250µl of 70% ethanol before a further 5 minute centrifugation at 13,000rpm. The pellet was then air dried and stored at -20°C until sequencing. Sequencing was performed by the Protein and Nucleic Acid Chemistry Laboratory at Leicester University. Sequence data was checked for identity via the Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) accessed on the internet. Lengths of intact sequence i.e. containing no N's, were scanned on the database. The most probable sequence match was taken as the result. N's occur when the sequencing software sees 2 or more peaks at the same point, or there is poor alignment of the peak, poor incorporation of the base at that point therefore giving a low fluorescence, or finally, there may be a compression of peaks, again the program forced to read more than 1 peak. The excess bases after the full length of the sequence has been read are accounted for by the sequencing program being run for a set length of time. Once the complete sequence is read, the program cannot read anything therefore designates N,s or occasionally picks up background fluorescence and designates a base.

## 4.3. RESULTS

*Radioactive Incorporation*

The amount of radioactive $^{3}$H-proline incorporation was compared on a per cell basis. Control cells showed an initial increase over the first 2 days with no great variation over the next 7 days, though by 10 days the amount of incorporation had doubled per cell compared to day 1. The conditioned medium treated cells however, showed fluctuation over the time period. Initial addition of ECCM caused a relatively large increase of incorporation of precursor compared to the control cells. By 2 days there had been a rapid decrease to less than half the incorporation seen initially. By 5 days, the incorporation was comparable to controls, but by 7 days, the incorporation per cell was only one fifth of the control value. At 10 days, the amount had increased again, but was still only just over half that of the control value (Table 4.2., Figure 4.1).

**Table 4.2.** Table to show average no. of cpm/cell.

| Day | No. of Viable Control Cells | Total cpm | Average cpm/cell | No. of Viable ECCM Cells | Total cpm | Average cpm/cell |
|---|---|---|---|---|---|---|
| 1 | $7.78x10^{4}$ | 110,580 | 1.42 | $8.93x10^{4}$ | 213,030 | 2.39 |
| 2 | $1.17x10^{5}$ | 303,210 | 2.59 | $1.08x10^{5}$ | 127,680 | 1.18 |
| 5 | $1.65x10^{5}$ | 369,300 | 2.24 | $1.46x10^{5}$ | 320,310 | 2.19 |
| 7 | $2.07x10^{5}$ | 425,130 | 2.05 | $3.2x10^{5}$ | 124,050 | 0.39 |
| 10 | $2.3x10^{5}$ | 736,530 | 3.2 | $6.3x10^{4}$ | 115,830 | 1.84 |

The percentage of radioactive precursor incorporated into high molecular weight proteins (including collagens) was also calculated. Control

cells showed a relatively low percentage of high MW protein incorporation over the first 2 days, but this increased to over half of the total incorporation by 5 days, then decreased to approximately one quarter of the total by 10 days. The conditioned medium treated cells however, showed a low, but constant, incorporation of radioactivity into high molecular weight proteins, the highest incorporation being just over 16% at 7 days of incubation (Table 4.3. & 4.4., Figure 4.1).

**Table 4.3.** Table to show average no. of cpm/cell of high molecular weight proteins.

| Day | Total viable cells (Control) | Total cpm in high MW proteins | Average high MW cpm/cell | Total viable cells (ECCM) | Total cpm in high MW proteins | Average high MW cpm/cell |
|---|---|---|---|---|---|---|
| 1 | $7.78x10^4$ | 16,830 | 0.216 | $8.93x10^4$ | 26,730 | 0.299 |
| 2 | $1.17x10^5$ | 37,110 | 0.317 | $1.08x10^5$ | 19,230 | 0.178 |
| 5 | $1.65x10^5$ | 208,200 | 1.262 | $1.46x10^5$ | 47,100 | 0.322 |
| 7 | $2.07x10^5$ | 234,180 | 1.13 | $3.2x10^5$ | 20,100 | 0.063 |
| 10 | $2.3x10^5$ | 206,130 | 0.896 | $6.3x10^4$ | 14,280 | 0.226 |

**Table 4.4.** Table to show percentage of radioactivity incorporated into high molecular weight proteins per cell.

| Day | Control average cpm (total) | Average cpm (high MW) | % | ECCM average cpm (total) | Average cpm (high MW) | % |
|---|---|---|---|---|---|---|
| 1 | 1.42 | 0.216 | 15.2 | 2.39 | 0.299 | 12.5 |
| 2 | 2.59 | 0.317 | 12.2 | 1.18 | 0.178 | 15.1 |
| 5 | 2.24 | 1.262 | 56.3 | 2.19 | 0.322 | 14.7 |
| 7 | 2.05 | 1.13 | 55.1 | 0.39 | 0.063 | 16.1 |
| 10 | 3.20 | 0.896 | 28.0 | 1.84 | 0.226 | 12.3 |

*Fluorography*

$^{3}$H-proline labelled collagen samples obtained over a 10 day period were examined by SDS-PAGE and fluorography (Figure 4.2). As such, there was an internal control for markers of type II and type X collagen as normal cultured chick embryo hypertrophic chondrocytes were used in the experiments (Berry *et al.*, 1992) The most notable feature is the visible decrease in type X collagen intensity in comparison to the type II collagen band after 7 days and the eventual disappearance of type X collagen after 10 days though a faint band of a slightly smaller molecular weight is present. Though there appeared only faint bands in the 2 and 5 day ECCM lanes, this was due to the fact that only a low number of counts were achieved in this particular sample, particularly for the 5 day sample, and therefore less than 20,000cpm was loaded onto the gel, however, this fluorogram emphasises the loss of type X collagen more clearly than other fluorograms. Other samples showed equal intensities for type II and type X. The intensity of the type II and type X bands appeared similar, indicating equal amounts of production, as is seen in control lanes. There generally remained a strong expression of a collagen migrating where type II would be expected to migrate (~110,000MW) in all lanes.

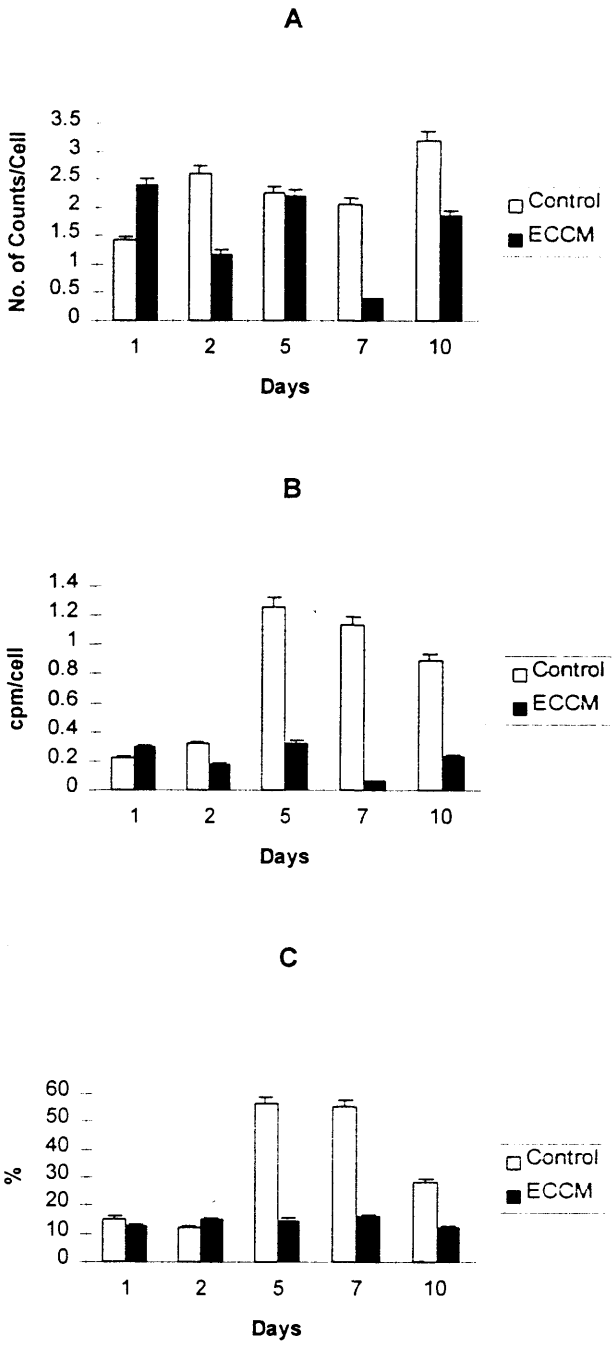



**Figure 4.1.** A. Comparison of radioactive incorporation (cpm/cell). B. Comparison of incorporation of radioactive precursor into high molecular weight proteins (cpm/cell). C. Percentage incorporation of radioactivity into high molecular weight proteins per cell. (Bars = 2SD)

*Immunoblots*

There is release of type II collagen into the medium by both control and ECCM treated cells at each time point over the entire 10 day period (Figure 4.3). Blots for type I collagen were extremely inconsistent. In two samples, there was no type I collagen at all. In another, type I was only present in control cultures up to day 7, and not present at all in ECCM cultures (Figure 4.4). A further blot showed type I expression up to 7 days in both control and ECCM cultures, then a disappearance at 10 days of culture (Figure 4.4). Type III collagen was expressed by both control and ECCM cultures up to day 7, then disappeared by day 10 (Figure 4.5). Type III collagen was examined in its unreduced state and therefore migrated high in gels and on blots.

*Collagen Immunocytochemistry*

*Type I Collagen:*

Control and ECCM treated chondrocytes stained negatively for type I collagen at 2 days, with a few individual cells staining positively at 5 days. At 7 and 10 days, some small fibroblastic colonies in control cultures were positive (Figure 4.6), the majority of the cells were still negative. ECCM treated cells were negative for type I collagen at 7 and 10 days (Figure 4.7).

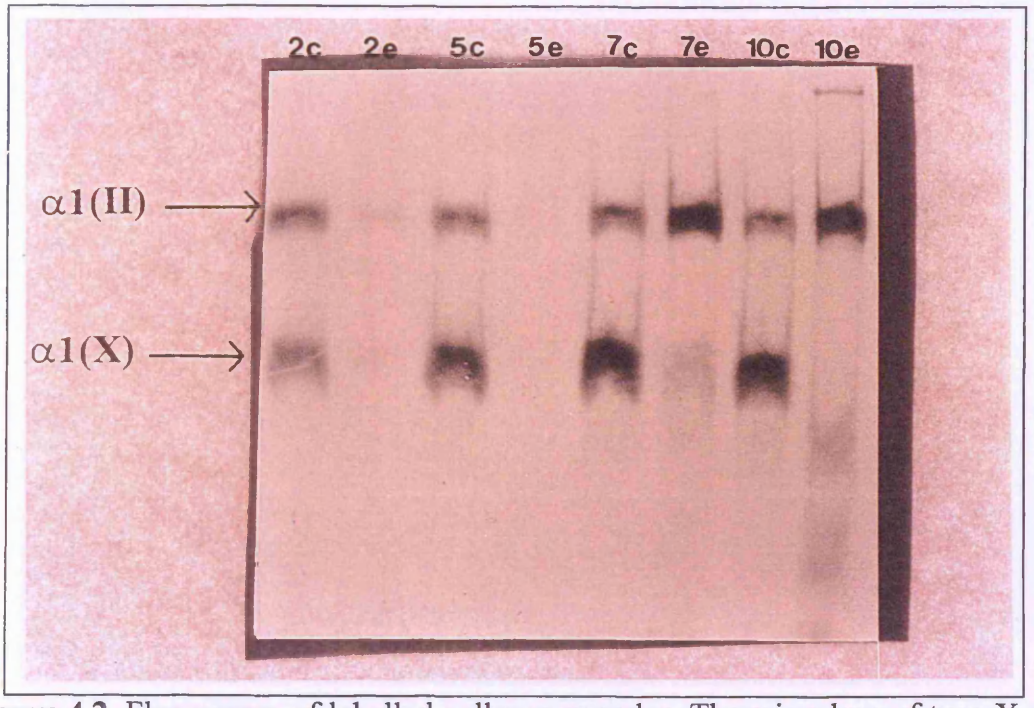



**Figure 4.2.** Fluorogram of labelled collagen samples. There is a loss of type X after 7 and 10 days of ECCM treatment, though there are indications of a slightly smaller molecule than type X in the 10 day ECCM lane. The lack of intensity in both 2 and 5 day ECCM lanes is explained by the low counts loaded onto the gel.

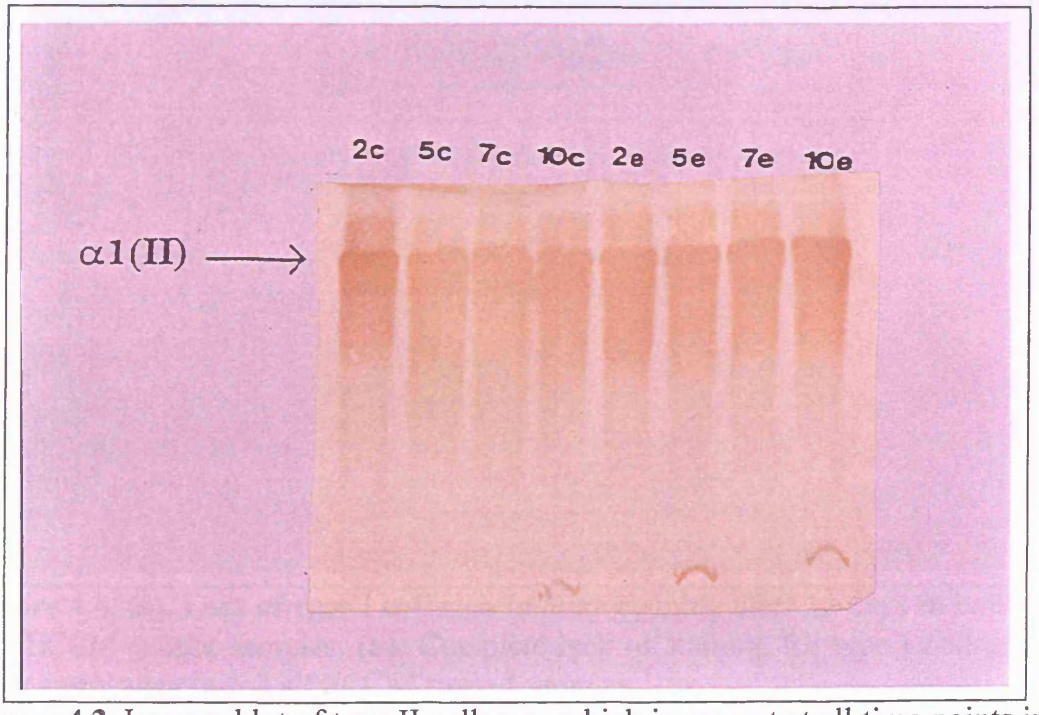



**Figure 4.3.** Immunoblot of type II collagen, which is present at all time points in both control and ECCM treated samples.

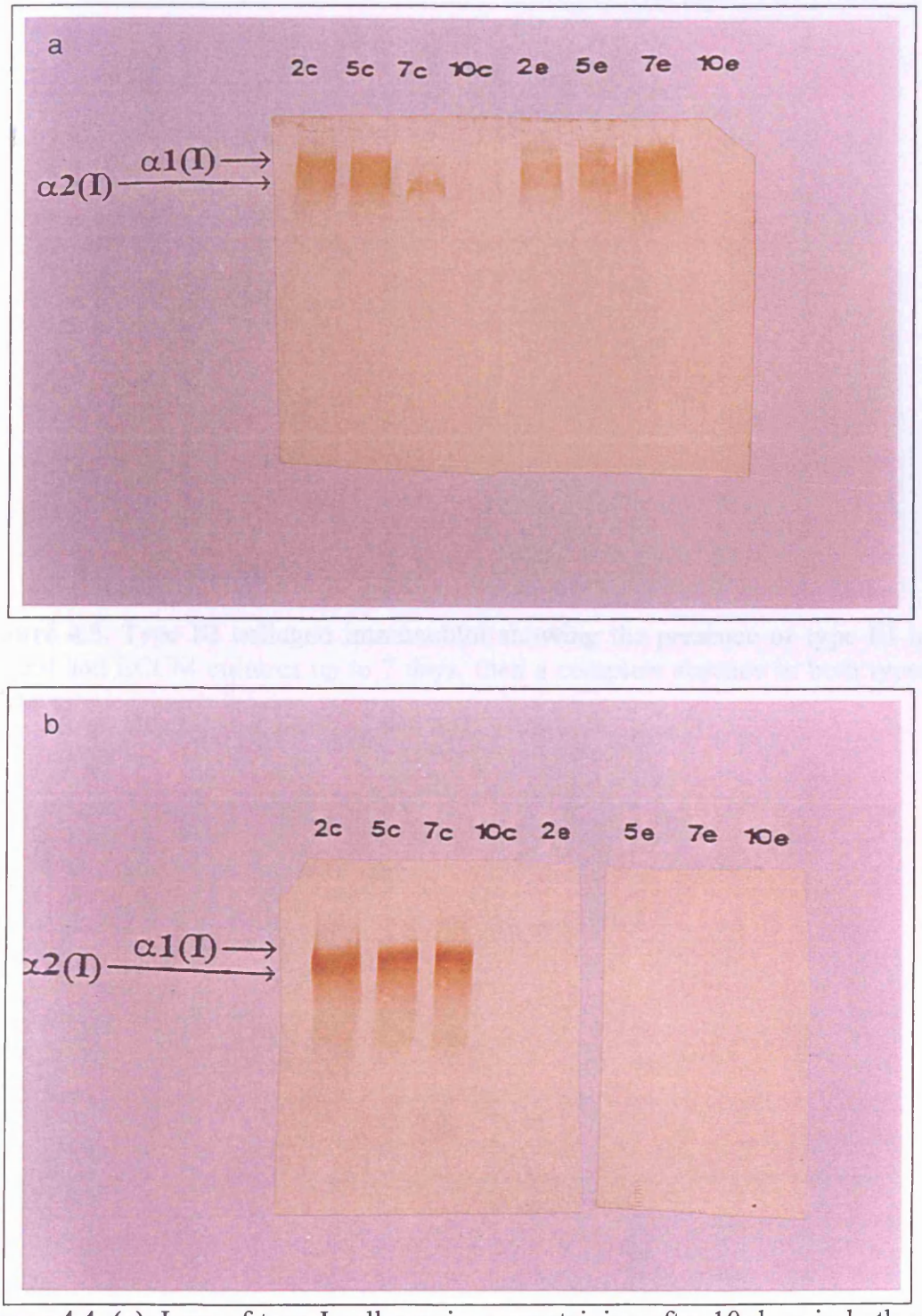



**Figure 4.4. (a).** Loss of type I collagen immunostaining after 10 days in both control and ECCM culture samples. **(b).** Complete lack of staining for type I collagen in 10 day control sample and all ECCM treated samples.

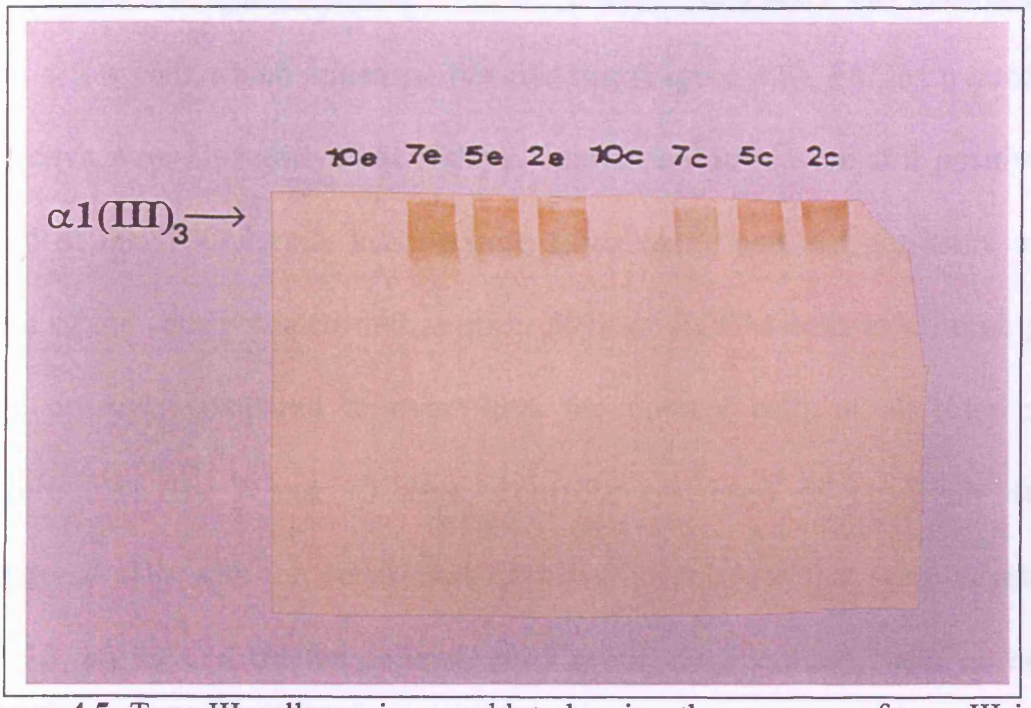



**Figure 4.5.** Type III collagen immunoblot showing the presence of type III in both control and ECCM cultures up to 7 days, then a complete absence in both types after 10 days.

*Type II Collagen:*

Type II collagen staining was very positive in 2 and 5 day controls, apart from a few cells which appeared fibroblastic (Figure 4.8). ECCM treated cells at 2 days were all positive. At 5 days, control cultures were still positive, but many of the ECCM cells had become fibroblastic and did not stain, a brief count of the cells revealed that approx. 50% of ECCM cells at all time points were positive, compared to over 90% for control cells at all time points. (Figure 4.9). By 7 and 10 days, controls consisted of positively stained polygonal cells with the occasional fibroblastic colonies that were negative. 7 and 10 day ECCM treated cells showed much degeneration, with no healthy chondrocytes present. The cells at both time points had a rounded morphology, though much smaller than freshly plated chondrocytes, and stained positively for type II collagen (Figure 4.10).

*Type III Collagen:*

2 and 5 day control cultures generally stained positively with the anti-type III collagen antibody. 7 and 10 day controls showed a mixture of positively and negatively stained cells. 2 day ECCM cultures showed a more variable staining pattern, with a number of negative cell clusters in amongst positive cells. By 5 days, the ECCM treated cells were completely negative for type III collagen, and the cells remained this way at 7 and 10 days (Figure 4.11).

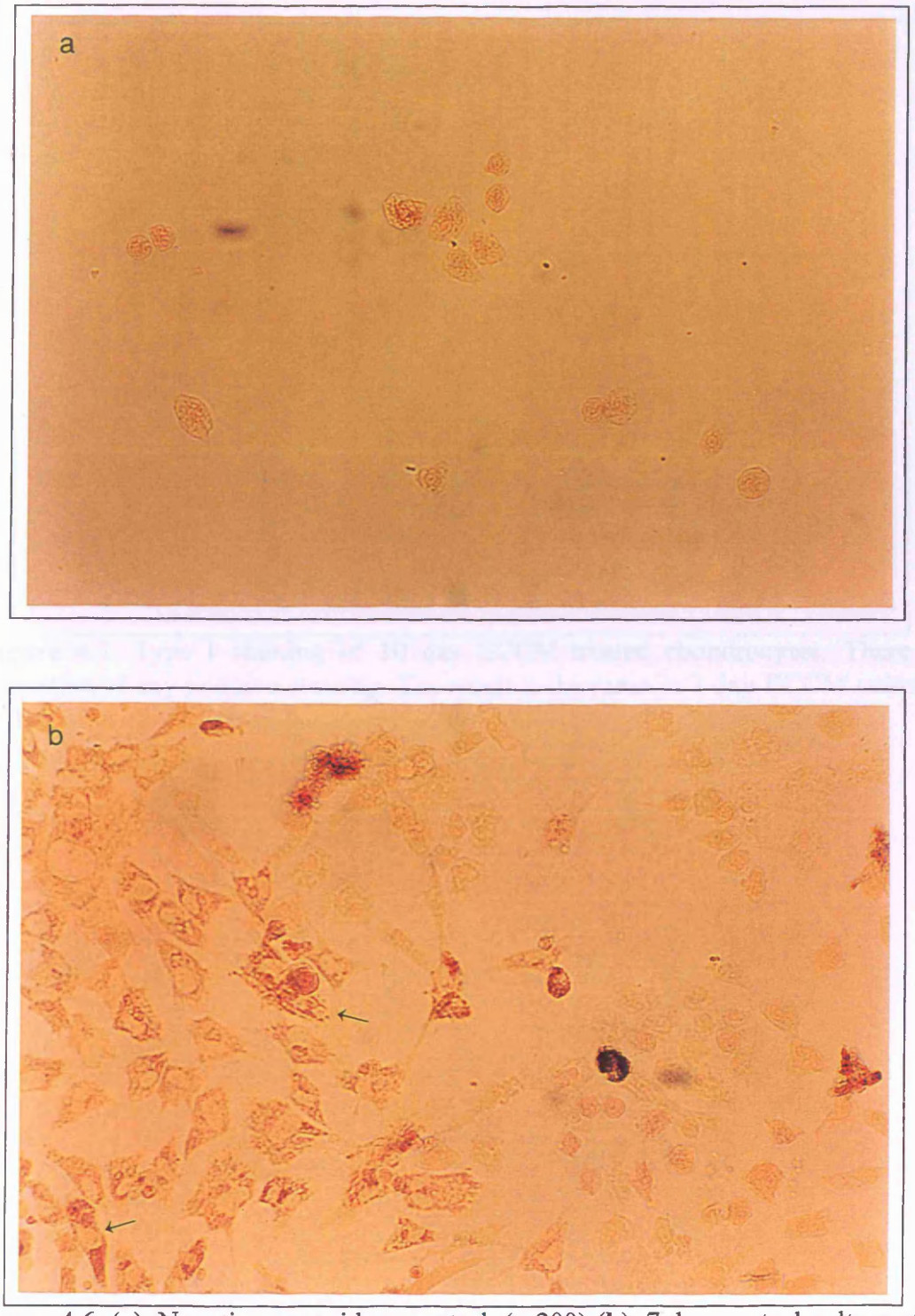



**Figure 4.6. (a).** Negative peroxidase control. (x 200) **(b).** 7 day control culture stained for type I collagen. There is an equal mix of positively and negatively staining cells. Cells with a more fibroblastic morphology tend to be the positive staining ones. (x 200).

**Figure 4.7.** Type I staining of 10 day ECCM treated chondrocytes. There is no indication of any positive staining. The result is the same in 7 day ECCM cultures. (x 200).

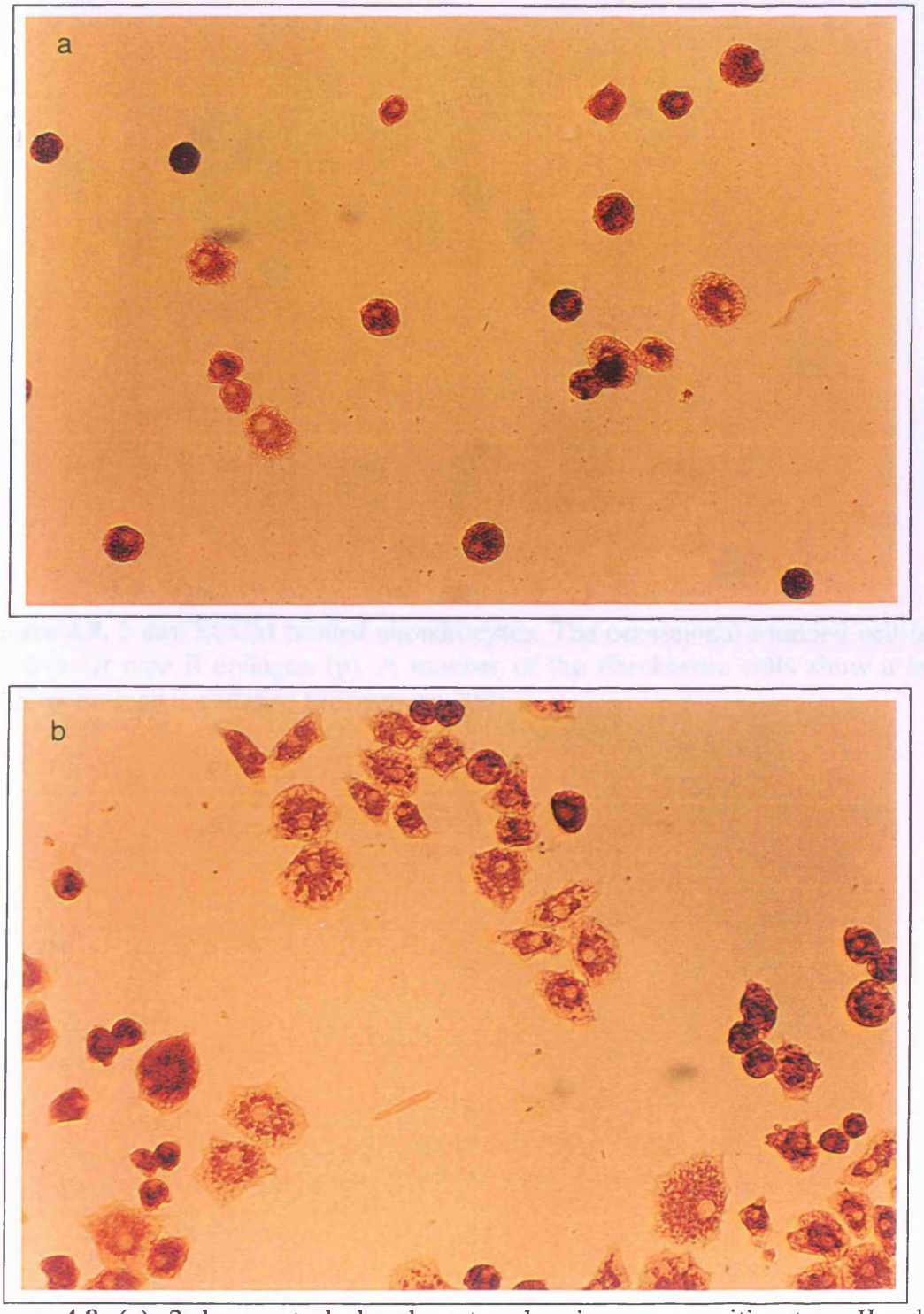



**Figure 4.8. (a).** 2 day control chondrocytes showing very positive type II collagen staining. (x 200). **(b).** 5 day control chondrocytes, though more polygonal, still showing intense positive type II staining. (x 200).

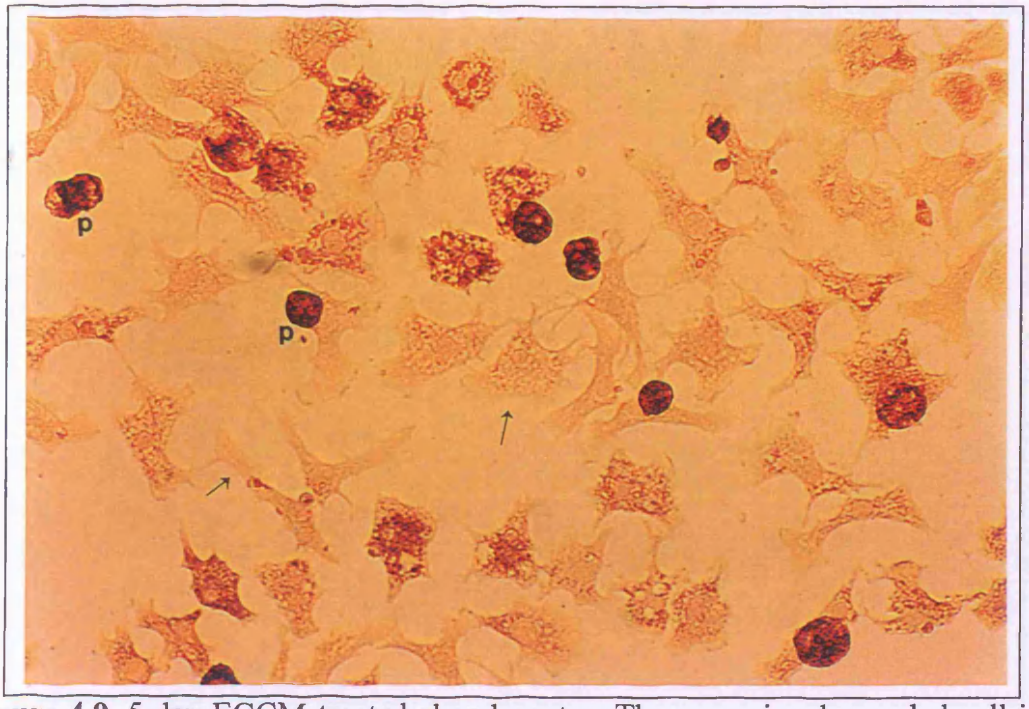



**Figure 4.9.** 5 day ECCM treated chondrocytes. The occassional rounded cell is very positive for type II collagen (p). A number of the fibroblastic cells show a lack of staining for type II collagen (arrows). (x 200).

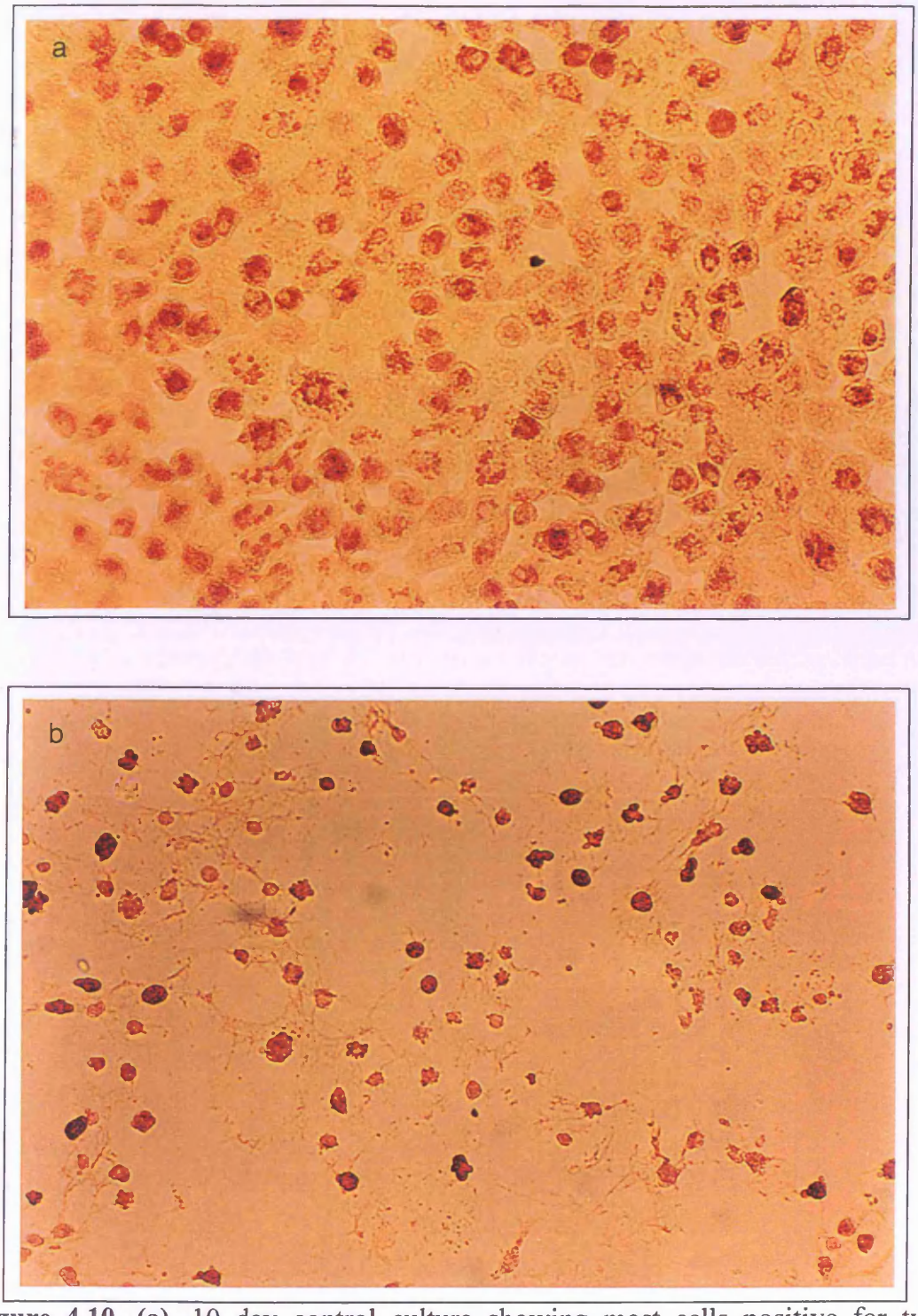



**Figure 4.10. (a).** 10 day control culture showing most cells positive for type II collagen. (x 200). **(b).** 10 day ECCM treated chondrocytes. Despite being very shrunken and degenerate, the cells stain very positively for type II collagen. (x 200).

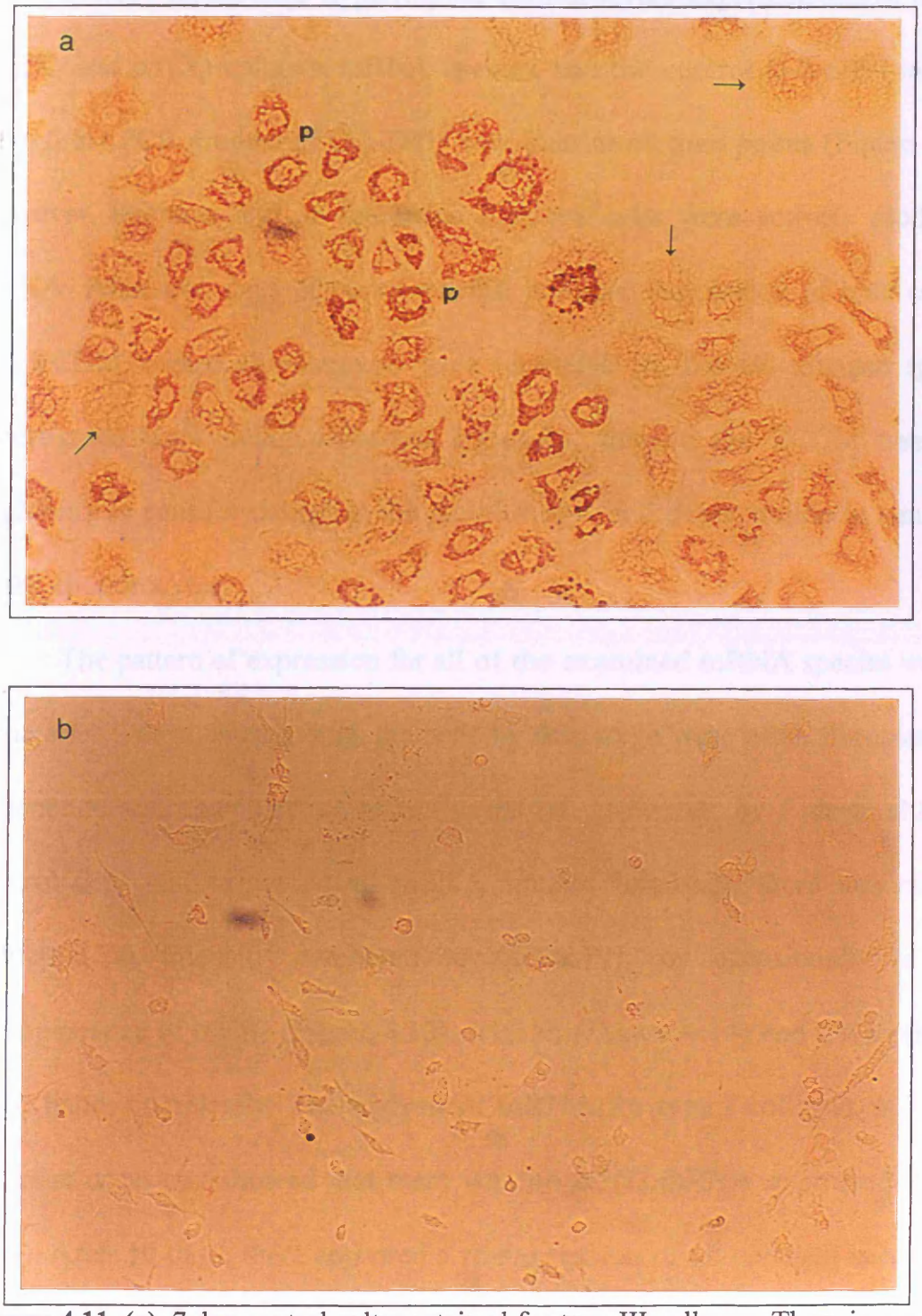



**Figure 4.11. (a).** 7 day control culture stained for type III collagen. There is a mixture of positively stained (p), and negatively stained (arrows) cells. (x 200). **(b).** 7 day ECCM treated culture, showing no type III staining. A similar result was seen in 5 and 10 day ECCM cultures. (x 200).

*RT-PCR of Collagen mRNA in Control and ECCM Treated Chondrocytes*

Freshly dissociated hypertrophic chondrocytes expressed α2(I), α1(II), α1(IX) and α1(X) collagen mRNA species and the control G-3-DPH mRNA, though the PCR product of G-3-DPH was faint at all time points (Figure 4.12). However, this was still an indication that the cells were actively producing mRNA. After two days of treatment, the collagen expression of both control and ECCM treated chondrocytes were identical, in that all collagen species investigated were being expressed intensely, despite the ECCM treatment beginning to cause a change in the morphology, and degeneration in a number of the chondrocytes.

The pattern of expression for all of the examined mRNA species was the same after 5 days, even though the cells by this stage were often fibroblastic or degenerate and contained no refractive ECM. However, by 7 days, although control cells still expressed all mRNA species intensely, there was either a reduction in intensity compared to G-3-DPH, or occasionally, a total disappearance of α1(II) (Figure 4.13), α1(IX) (Figure 4.14) and α1(X) (Figure 4.15) bands completely. The analysis of mRNA for type I collagen, at 7 days, on most occasions showed that there was no α2(I) mRNA expressed (Figure 4.16). After 10 days, there appeared a re-expression of all collagen species at a relatively high band intensity (Figure 4.13 to Figure 4.16).

Sequencing of the PCR products (Figure 4.17 & 4.18) and subsequent analysis on the NCBI genetic database showed that the primers did amplify the correct

cDNA species from the RT-PCR reaction. All sequences were matched by the BLAST database as being the most probable sequence match to the respective chick collagen gene.

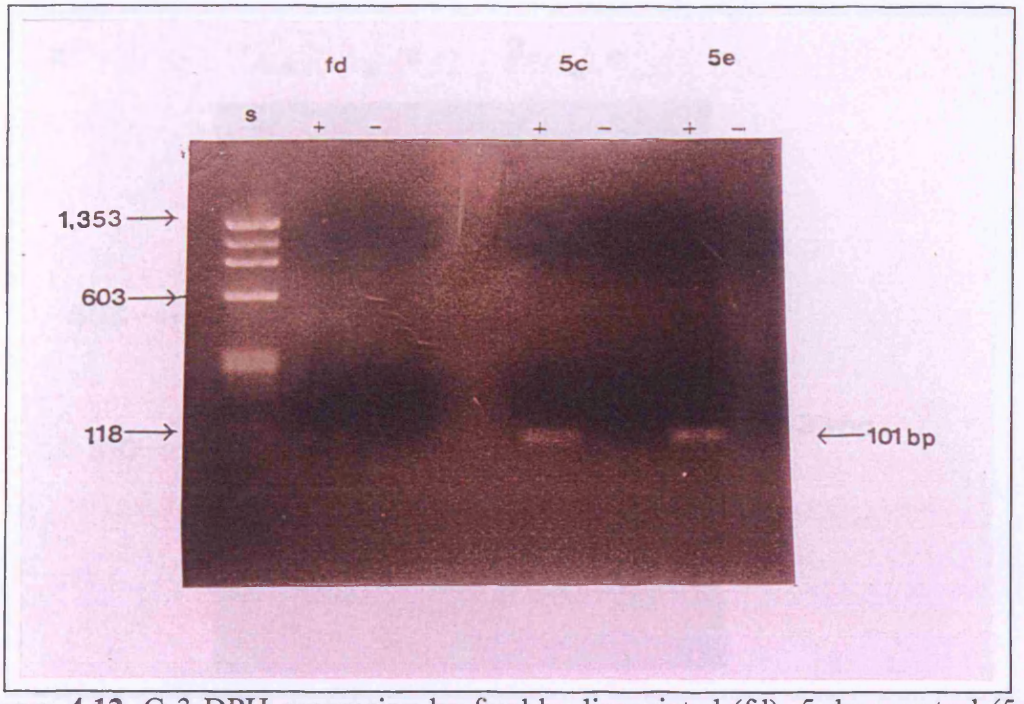
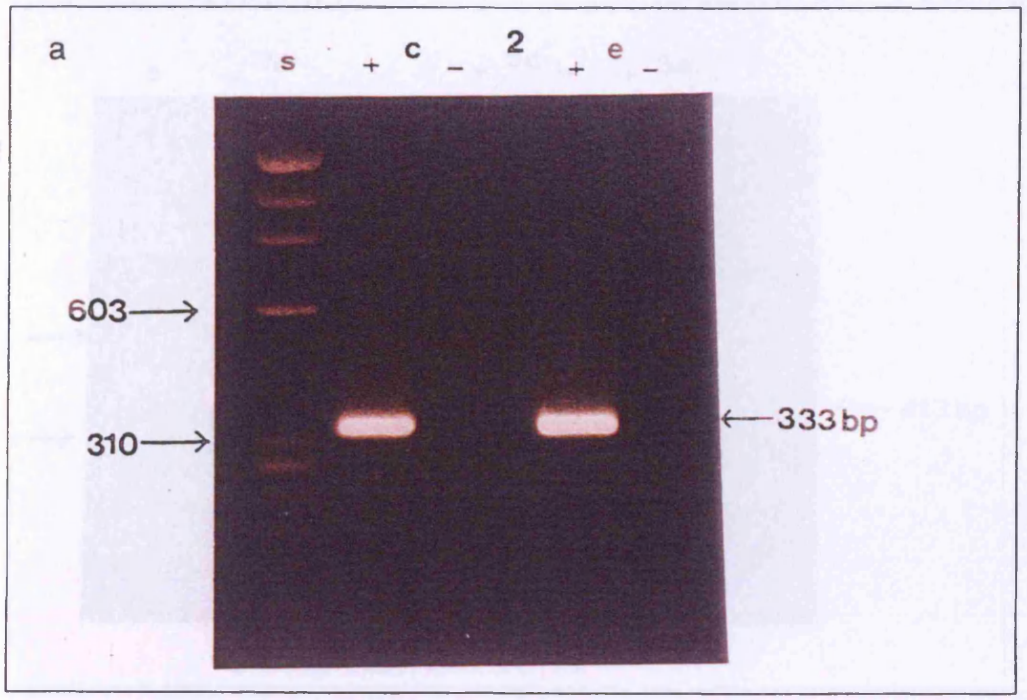



**Figure 4.12.** G-3-DPH expression by freshly dissociated (fd), 5 day control (5c) and 5day ECCM (5e) chondrocytes. + is the reaction with reverse transcriptase, - is the reaction without reverse transcriptase. All 3 samples show faint staining for G-3-DPH, the same is seen in all control and ECCM samples.



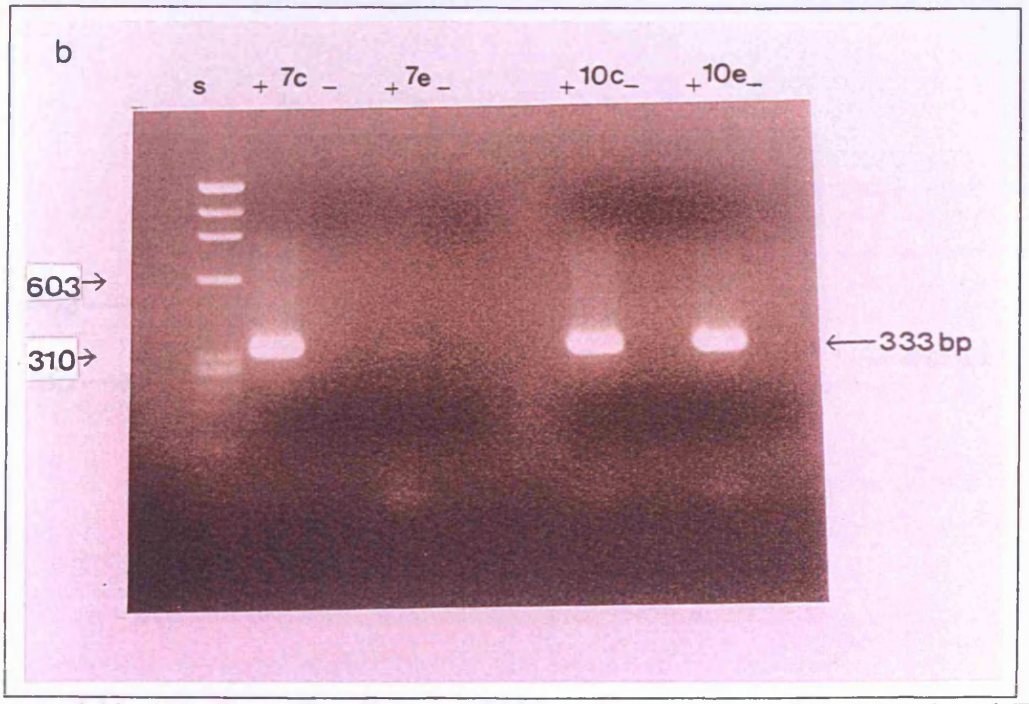




**Figure 4.13.** **(a).** Type II collagen mRNA expression in 2 day control and ECCM treated cultures. **(b).** Type II collagen mRNA expression in 7 and 10 day control and ECCM treated cultures. There is a distinct loss of intensity in the 7 day ECCM sample, but the intensity is restored in the 10 day sample.

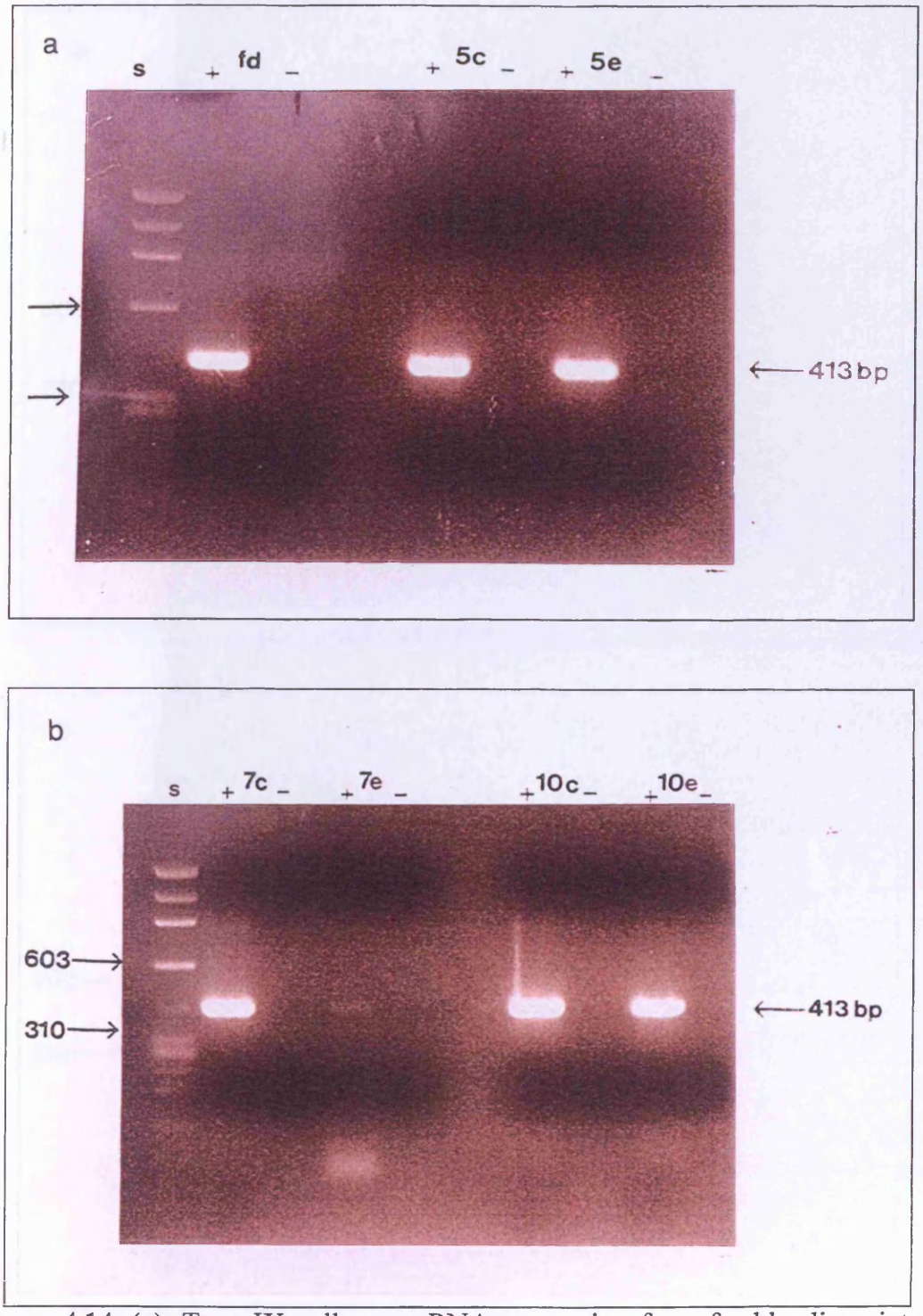



**Figure 4.14. (a).** Type IX collagen mRNA expression from freshly dissociated (fd) and 5 day control and ECCM treated cultures. Arrows on left indicate 603 and 310 base pair markers as seen previously. **(b).** Type IX collagen mRNA expression in 7 and 10 day control and ECCM treated cultures. As for type II mRNA, there is a distinct loss of intensity in the 7 day ECCM sample, which is restored after 10 days.

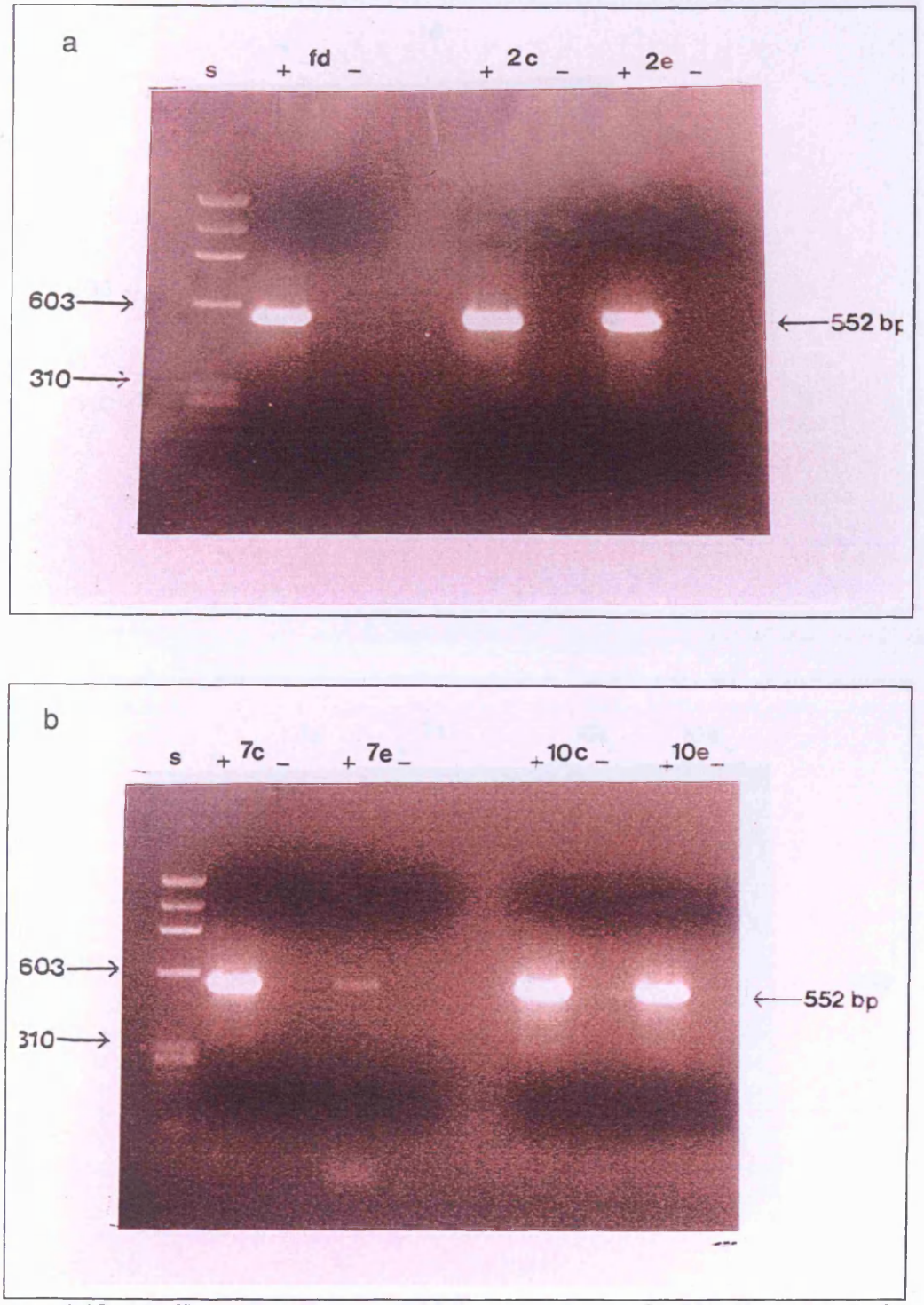
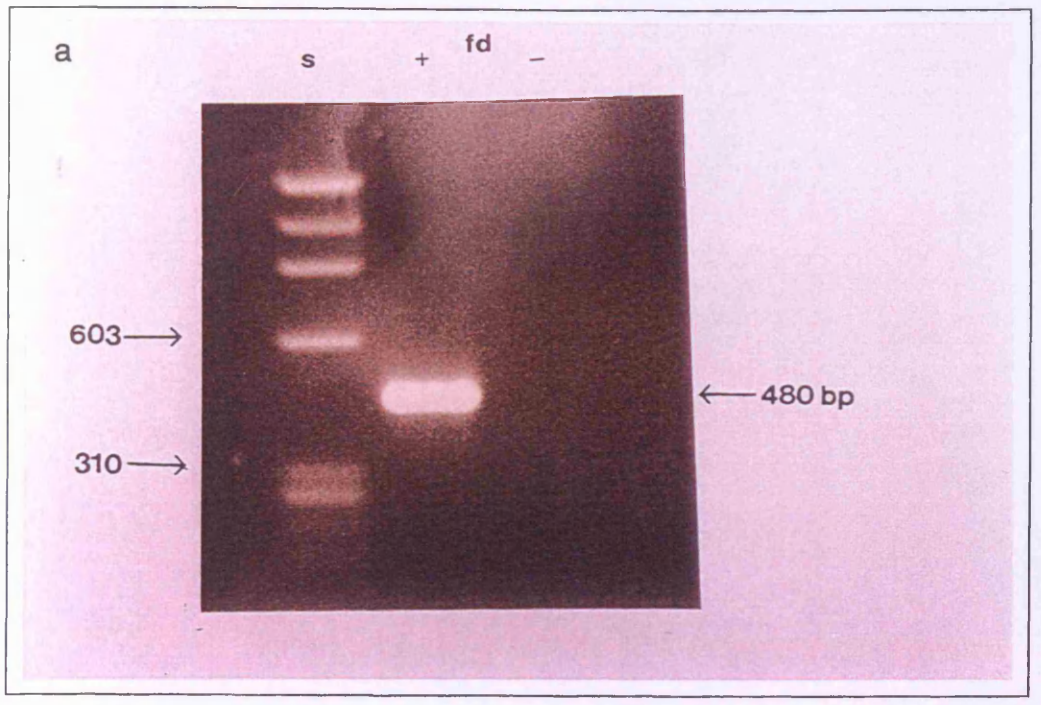



**Figure 4.15. (a).** Type X collagen mRNA expression in freshly dissociated (fd) and 2 day control and ECCM treated cultures. **(b).** Type X collagen expression in 7 and 10 day control and ECCM cultures. Once again, there is a loss of intensity in the 7 day ECCM sample which is restored after 10 days.



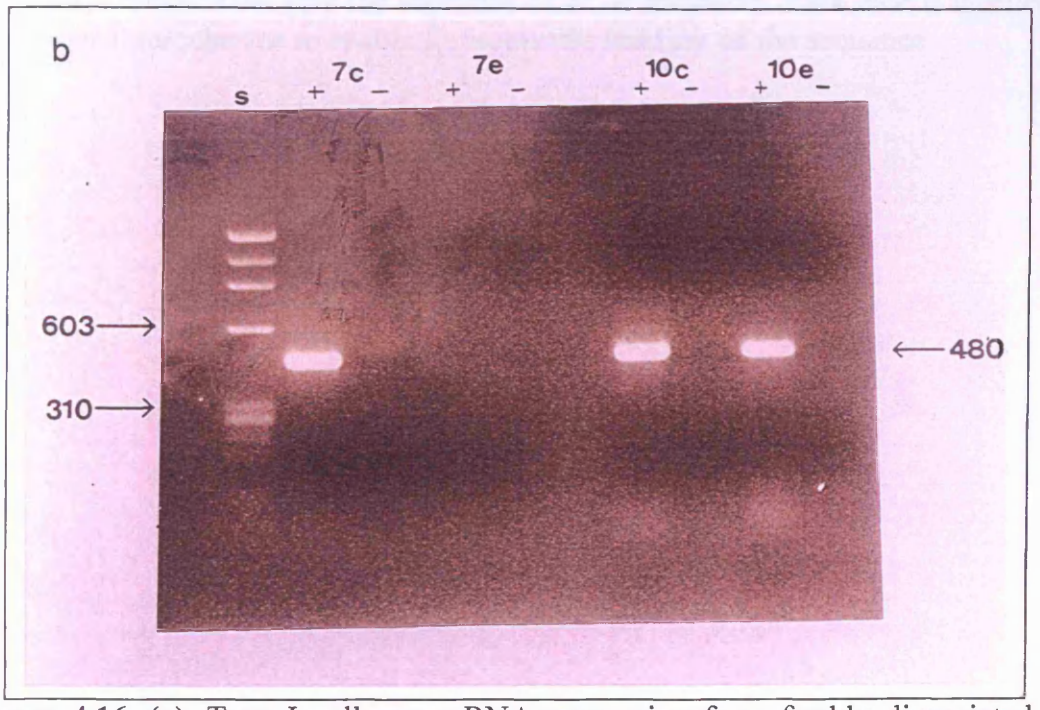
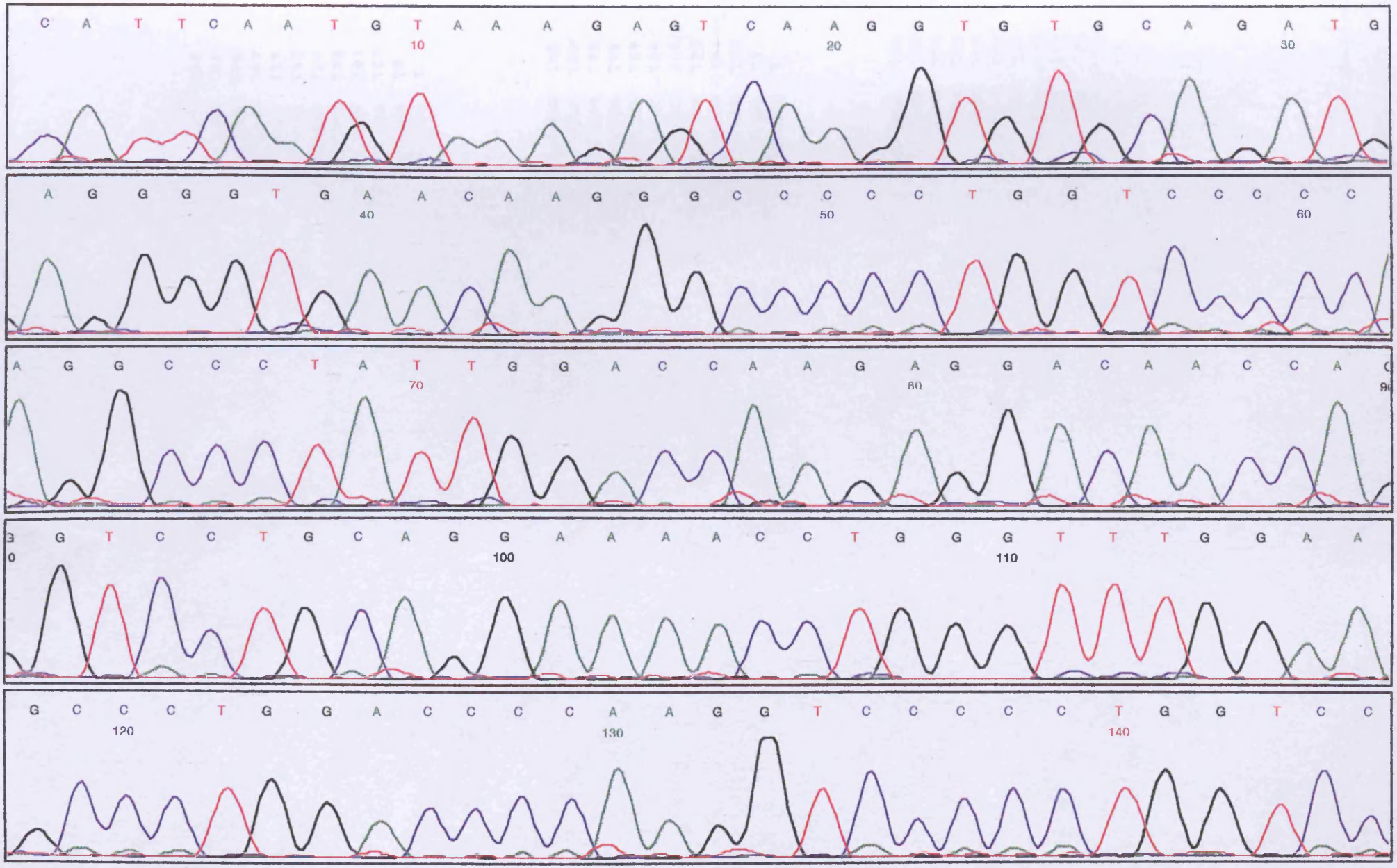




**Figure 4.16. (a).** Type I collagen mRNA expression from freshly dissociated cells. **(b).** Type I collagen mRNA expression from 7 and 10 day control and ECCM treated cultures. There is a complete lack of type I mRNA expression after 7 days of ECCM treatment, which is restored after 10 days.

**Figure 4.17.** Example of sequencing chart of the first part of the α1(X) cDNA (overleaf). These charts give the sequence of PCR products. Each base is labelled with a different fluorochrome to enable fluorometric reading of the sequence.


C A T T C A A T G T A A A G A G T C A A G G T G T G C A G A T G
10
20
30
A G G G G T G A A C A A G G G C C C C C T G G T C C C C C
40
50
60
A G G C C C T A T T G G A C C A A G A G G A C A A C C C A
70
80
G G T C C T G C A G G A A A A C C T G G G T T T G G A A
100
110
G C C C T G G A C C C C A A G G T C C C C C T G G T C C
120
130
140

a

```
   1  TNCCGTTCTC AGCCTTGNAC AGTGCCCTTG AAGTGTCCGT GTGTAGAATC ATATTTGAAC  60
  61  ATGTAAACCA TGTAGTGCAG ATCNCCNTGG GCCCCGTGGC GTGCCTGGCC NCAATGGTCC 120
 121  TCCTGGCTGA GANCGGTCNN NCAGGTCCAN TGNGGCGANC GCACAGNGGA AAGGGGGGCC 180
 181  CNGANGNGTT CCAGGCGGAC GAGTGANGGG GTCGNCCGGG ACNGGTCNNG NCNCGCAGGT 240
 241  GCNNCCGCGN AGGGGCCNGG NCNCGGGCAN GGGGGGCCCC NCGGGGNNGN NGGGGGTNGG 300
 301  NCCNGNCGGC GGCNGGGNCN CNGCGGGGNN NCCNCGGGNN NGCNGGGGCN CANTGGGGGC 360
 361  NTNCCGGGNA GGNGCGNGGG CGGNCGCCGT GCGTCCNCCC TGGGCNCGNC NGGGNCNNNG 420
 421  CCTNGTNCCC CCCNCGGGNG GGGGGGCNNG CCCNNGNCNG NCTNNNCGGG NCGNNCCTGN 480
 481  NNNGNGCCNG CGGNCCNNGG CGGGCCCGNG GGGGGCGCGC CCGCGCGCGC GNGGGGGGNT 540
 541  TGCGGGNNNN NNNNCCCNGG NGCCGCNNNC NNGGNCGNGN GCCNGCTCGN CNGCCTGGNT 600
 601  GGGCCCGGGG GCNGGGNNGN GGCTCGNNGN NCNCNNNCGN GGGCGGGCGG GGGGGNGGNC 660
 661  NNGGNCGGNC CCNGGGGNGG GCCGNGANNN TNCNGGGCNN NNCGNGNGNC TGGCGCNCCT 720
 721  CCTNGNGGCN CCGCANCCNG GNNCGGNNNT NGGNNNCNNT T                      780
```

b

```
   1  GCGTCCTGCA GGTAATAGCC AACCACGTCC GGGGGCTCTG CAACACAAGG AGTCTGCATG  60
  61  TCTAGCAAGT AGACATGCTC AGCTTTGTGG ATACGCGGAT TTTGTTGCTG CTCGCAGTAA 120
 121  CTTCATACCT AGCAACAAGC CAACATGTGA GTGAGGCATC TGCAGGGCGG AAGGGCCCTA 180
 181  GAGGAGACAA AGGGCCACAG GGAGAAAGGG GTCCACCAGG TCCACCAGGC AGAGATGGTG 240
 241  AAGACGGTCC ACCAGGTCCT CCAGGCCCCC CTGGTCCTCC AGGTCTTGGC GGAAATTTTG 300
 301  CTGCTCAGTA TGATCCATCT AAAGCGGCTG ACTTTGGCCC CGGACCTATG GGTTTAATGG 360
 361  GACCTANAGG CCCACCTGGA GCATCTGGAC CTCCTGGCCC TCCTGGGTTT CAAGGTGTTC 420
 421  CTGGTGAGCA TNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNN 480
 481  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNN 540
 541  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNN 600
 601  NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNN 660
 661  NNNNNNNNNN NNNNNNNNTT TTTTTTTT                                     720
```

c

```
   1  TGNANGTCAC CTACGGCNAT GANAACCTGT CCCCCAACAC CGCCAGCATC CAGATGACCT  60
  61  TCCTGCGCCT CCTGTCCACC GAGGGCTCCC AGAACNTCAC CTACCACTGC AAGAACAGCA 120
 121  TCGCCTACAT GGACNAGGAG ACGGGCAACC TGAAGAAAGC CATCCTCATC CAGGGATCCA 180
 181  ACGACNTGGA GATCAGAGCC GAGGGCAACA GCAGGTTCAC CTACAGCGTC TTGGAGGACG 240
 241  GCTGCACNAA ACACACTGGC AAATGGGGCA AGACGGTGAT CGAGTACCGG TCGCAGAAGA 300
 301  CCTCGCGCCT GAATCNCCAC NTCNTNNGTT CCCNGNCNGA CGNNNGGNTT CANCGGGNCN 360
 361  CACGTCNCCA CNTNGATNNN GGNGACGCCG TTNTNCCNNT GGNANGTGAG TTTGGGGTNC 420
 421  CTCNGTNNNA GGNNGTNNNG ANGGCNNCNG GGTNCNNNNG NNTNTGNGGA NNNGNCTNAT 480
 481  NCNCNTNNCN NNTNNGNTNN NGNNNNNTNT NNNNCTNNCN NNNNNGNTTN NNTNNNNNNN 540
 541  NNNNNNNNNT NNNNNTGNNN NNNNNNNNNN NTTNNNNNNNT NNNNNNNNNN NNNNNNNTN 600
 601  NNTTNNNNNN GNNNNNNNNN NNNNTNNNNN NNNNNNNNNN NNNNNNNNNN NTTNTTTTTT 660
 661  TT                                                                  720
```

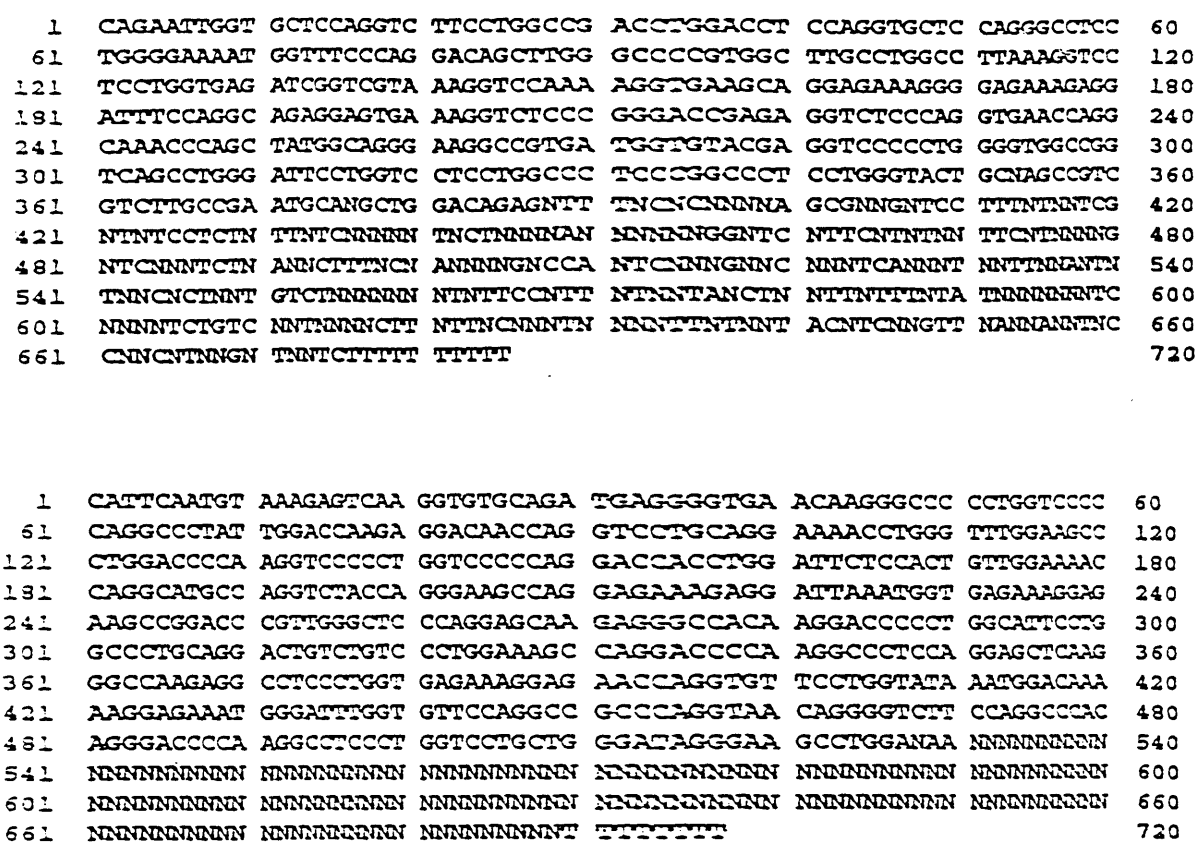


Figure 4.18. Sequence data from the dRhodamine sequencing reaction performed on the PCR products of the primer sets for (a). G-3-DPH, (b). α2(I), (c). α1(II), (d). α 1(IX), (e). α1(X).

## 4.4 DISCUSSION

Despite the obvious morphological and apparently necrotic changes in hypertrophic chondrocytes treated with bovine ECCM, there is no outstanding qualitative difference in terms of their collagen expression at either the protein or the mRNA level though occasional differences do occur. Quantitatively though, on a per cell basis, radiolabelling of collagens showed that ECCM treated cells produced much less collagenous protein than their control counterparts, indicating that there is a decrease in the rate of collagen production by the chondrocytes during treatment. This may in part be accounted for by the apparent loss of type II collagen production in approximately half of the conditioned medium treated cells. Though this also implies that approximately half still produce type II.

At the cellular level, immunostaining of ECCM treated cell cultures showed a constant presence of type II collagen throughout the culture period, further shown by the release of type II collagen into the medium, though as just mentioned, not by all cells. Type II collagen expression is indicative of the chondrocytic phenotype (Miller & Matukas, 1969; Solursh *et al.*, 1982; Horton & Hassell, 1986). Many of the treated cells therefore maintain the chondrocytic phenotype despite the change in morphology and the degenerative changes occurring

Chondrocytes have been shown to express type I and occasionally type III collagen under various conditions, particularly when dedifferentiating (Mayne *et al.*, 1976; Gauss & Muller, 1981; Goldring *et al.*, 1988; Gerstenfeld

*et al.*, 1989; Kirsch *et al.*, 1992; Dietz *et al.*, 1993; Bonaventure *et al.*, 1994; Mallein-Gerin *et al.*, 1995). Cultured chick embryo hypertrophic chondrocytes also express both type I and III collagen, certainly under control conditions. The presence of both was seen in the culture medium, though the deposition of these collagens is not particularly detected at the cellular level. The control of collagen expression has often been shown to be at the transcriptional level (Mallein Gerin *et al.*, 1990; Thomas *et al.*, 1990), however, here, the mRNA transcripts (for type I and also type X collagen) are still present at the late stages of treatment even though indications from fluorography, immunoblotting and immunocytochemistry indicate that these two collagens are not present at the protein level. Further investigation with a primer for type III collagen is required to determine the level of control of this particular collagen.

A change in morphology from a polygonal chondrocyte to an elongated fibroblastic cell is usually associated with a switch in collagen expression from type II to type I (Mayne *et al.*, 1976; Castagnola *et al.*, 1986; Kirsch *et al.*, 1992; Bonaventure *et al.*, 1994). The change to a fibroblastic morphology of chick embryo hypertrophic chondrocytes, when treated with bovine ECCM does not appear to be linked to a specific switch to type I collagen expression. This then indicates that the process occurring in the ECCM treated cells is different to the dedifferentiation of chondrocytes, and may not be the same process that is normally associated with chondrocytes in culture (Mayne *et al*, 1976; Kirsch *et al*, 1992; Bonaventure *et al*, 1994).

There is a persistence of type II collagen expression by the ECCM

treated chondrocytes, even at late stages of treatment, i.e. 10 days. These late cells therefore, phenotypically at least, can be classed as chondrocytes, in that they express type II collagen, despite having changed to a fibroblastic form, before shrinking and rounding up again. The rounded morphology has been associated with the chondrocyte phenotype (Archer *et al.*, 1990; Bonaventure *et al.*, 1994), though there is evidence that morphology is not a determining factor of a chondrocytes phenotype and non-rounded chondrocytes have the ability to express cartilage specific collagens (Horton & Hassell, 1986; Mallein-Gerin *et al.*, 1991). The cells do however, lose their hypertrophic nature seen by the loss of type X collagen, which is associated with the hypertrophic phenotype (Kielty *et al.*, 1985; Schmid & Linsenmayer, 1987; Kirsch *et al.*, 1992).

# CHAPTER 5

# A Study of the Mechanism of Cell Death in Conditioned Medium Treated Chondrocytes

## 5.1. INTRODUCTION

There is uncertainty about the fate of the chondrocyte during vascular invasion of hypertrophic cartilage. It is generally accepted that hypertrophic chondrocytes are terminally differentiated cells and simply die during the invasion process (see Roach *et al.*, 1995) however, recent work has provided evidence for apoptosis of some chondrocytes along the invasion rim during hypertrophic core invasion (Gibson *et al.*, 1995b). From an opposite viewpoint, Galotto *et al.*, (1994) provide evidence for further differentiation, *in-vivo*, of hypertrophic chondrocytes to an osteoblast-like cell around the vascular canals of developing bones. This suggested that there is direct participation of hypertrophic chondrocytes in bone formation. Roach (1992) has also observed this phenomenon, though more recent work by Roach *et al.*, (1995) has shown that both differentiation and apoptosis occur, via asymmetric division of a parent cell, one daughter cell rapidly dying, the other remaining viable and subsequently dividing to produce osteoblast-like cells.

There is an obvious degeneration of hypertrophic chondrocytes during culture in ECCM. Along with the study of phenotypic changes, it is important to elucidate the ultimate effect that the conditioned medium has on the cells. There are two distinct forms of cell death, necrosis and apoptosis, which can be determined morphologically (Cohen, 1996). Necrosis is characterised by swelling and bursting of the cell due to a loss of its ability to maintain homeostasis (Cohen, 1996). Apoptosis involves a number of changes, including ruffling or blebbing of the cell membrane, condensation of chromatin, loss of

cell and nuclear volume and fragmentation of DNA (Koopman *et al.*, 1994; see Cohen, 1996 for review of apoptosis). The DNA fragmentation is apparently due to random double stranded-breaks in the linker region between nucleosomal cores (Cohen, 1996) resulting in oligonucleosomal fragments of 150-200 base pairs (Zenmyo *et al.*, 1996). This can be easily demonstrated as a ladder effect on agarose gel electrophoresis (Wolfe *et al.*, 1996). In this chapter whole cell digests of chondrocytes were tested for the presence of DNA fragmentation.

At an early stage of the apoptotic process, cells lose their phospholipid membrane asymmetry, and expose phosphatidyl serine extracellularly, while maintaining membrane integrity (Koopman *et al.*, 1994; Martin *et al.*, 1995; Vermes *et al.*, 1995; Cohen, 1996; van Engeland *et al.*, 1996). Proteins of the annexin family bind tightly to anionic phospholipids (Rao *et al.*, 1992). One member of this family, annexin V, has a high affinity for phosphatidyl serine (Vermes *et al.*, 1995; van Engeland *et al.*, 1996). When conjugated to FITC, annexin V has been used in flow cytometric methods to detect apoptotic cells (Koopman *et al.*, 1994; van Engeland *et al.*, 1996).

FITC conjugated annexin V, in conjunction with propidium iodide (PI) (to discriminate necrotic cells (Vermes *et al.*, 1995)) was applied here to investigate the presence of apoptotic cells in hypertrophic chondrocytes during culture in ECCM by the use of flow cytometry.

## 5.2. MATERIALS AND METHODS

*DNA Fragmentation*

The presence or absence of DNA fragmentation was determined by a whole cell digestion method, described by Wolfe *et al.*, (1996). Hypertrophic chondrocytes, after both 5 and 10 days of control and ECCM treatment were trypsined with 1x Trypsin/EDTA solution (Gibco), centrifuged at 1400 rpm, counted on a haemocytometer, then rinsed in PBS and pelleted. The cell pellet was resuspended in 15µl of sterile distilled water, 9µl of 5x loading buffer (Appendix II), and 6µl of RNase solution (Appendix II) and incubated at room temperature for 30 minutes to lyse the cells and degrade the RNA. This solution was then loaded onto a 1.8% agarose gel (ICN Genetic Technology Grade) along with ϕX174/Hae III standard markers (Promega), containing ethidium bromide (50pg/ml). A digestion gel containing 0.8% agarose plus 50µl/ml of proteinase K solution (25mg/ml) (Sigma) was inserted above the sample lanes. The gel was run at 2V/cm length for 1 hour then at 10v/cm length for a further 1 hour before examining on a UV light box.

*Annexin V Binding*

Chondrocytes from freshly dissociated stage 36 (10 day) hypertrophic zones and from 2, 5, 7 and 10 days of culture in control medium or ECCM were trypsinised, pelleted, rinsed in PBS and re-pelleted before being treated in one of four ways below. One set of cells was also treated with staurosporin overnight, to induce apoptosis, and were treated in the same manner:

i) Resupended in 400µl of PBS

ii) Resuspended in 400µl of a 0.5µg/ml solution of (PI)

iii) Resuspended in Annexin V-FITC solution (Bender MedSystems) diluted as per manufacturers protocol, incubated at room temperature for 10 minutes, rinsed then resuspended in manufacturers binding buffer.

iv) Resuspended as above with Annexin V plus PI.

These cell suspensions were analysed by flow cytometry on a Becton Dickinson FACScan.

Analysis of the results was performed on Lysis II software (Becton Dickinson) using dot plots. The data was achieved by a number of steps (Figure 5.2.):

i) Measurement of forward scatter height (FSC-H) against side scatter height (SSC-H) showed a relatively discrete population of cells and another population that appeared much less discrete.

ii) The discrete population was gated (R1), and PI only labelled cells from this gate were analysed on FSC-H against the FL-3 detector which measures PI fluorescence.

iii) Those cells showing no increase in FL-3 fluorescence (and therefore no PI fluorescence) were further gated (R2) so that only viable cells were analysed for annexin V binding.

iv) Unlabelled cells from R2 were plotted in FL-1 (the detector which measures FITC fluorescence) against FL-3 to show background fluorescence of unlabelled cells. Quadrants were then plotted at the upper limits of FL-1 and

FL-3 fluorescence. Anything above the limit in subsequent analysis of background FL-1 fluorescence was deemed to be annexin V positive (in theory there should be no shift in FL-3 as all PI positive cells have been gated out already, however, there is often some overspill from FL-1 into FL-3 (L. Reeve, personal communication).

v) The percentage of shifted cells is presented as an average of two values from annexin V only labelled cells and from cells labelled with both annexin V and PI (Table 5.1).

## 5.3. RESULTS

*DNA Fragmentation*

Due to the small number of cells available, a whole cell digestion method, as opposed to DNA extraction methods, was preferred to look for internucleosomal cleavage. Degenerate hypertrophic chondrocytes from both 5 and 10 days show no sign of DNA laddering, only a single band of DNA of high molecular weight, much greater than 1,300bp, which is the first standard marker (Figure 5.1).

*Flow Cytometry*

30% of freshly dissociated hypertrophic chondrocytes show a level of FITC fluorescence above background, indicating that they had bound the annexin V-FITC. Staurosporin treatment of cultured control chondrocytes causes an almost threefold increase in FITC fluorescence over freshly dissociated cells. Staurosporin is an inducer of apoptosis (Leist *et al.*, 1997). The results with the control and conditioned medium treated cells however, are not so revealing. Although 2 day controls only show a slight increase in annexin V binding above freshly dissociated cells, by 5 days almost 90% of the cells bind annexin V-FITC, which by 7 days has decreased again to 35% only to rise again at 10 days to 92%. In both the 5 and 10 day control cultures, the percentages of apoptotic cells are higher, even than the numbers induced by staurosporin. Certainly at 5 and 10 days, in control cultures, there is no morphological indication that this amount of cells are undergoing apoptosis and equivalent

cultures not used for analysis survive for many days beyond these times. Even at 2 and 7 days of control culture, there is no visible indication that over a third of the cells are undergoing apoptosis. ECCM cultures tend to show an increased percentage of FITC positive cells at the equivalent time points except at 10 days when only 73% of cells are positive, compared to 92% in control culture (Table 5.1, Figures 5.3-5.6).

**Table 5.1.** Table to show percentage of annexin V positive cells after treatment.

| Treatment | Average % of R2 cells showing FITC fluorescence |
|---|---|
| Freshly Dissociated | 29.25 |
| Staurosporin | 77.94 |
| 2 Day Control | 36.7 |
| 2 Day ECCM | 52.4 |
| 5 Day Control | 89.6 |
| 5 Day ECCM | 98.5 |
| 7 Day Control | 35.5 |
| 7 Day ECCM | 67.3 |
| 10 Day Control | 92.1 |
| 10 Day ECCM | 73.8 |

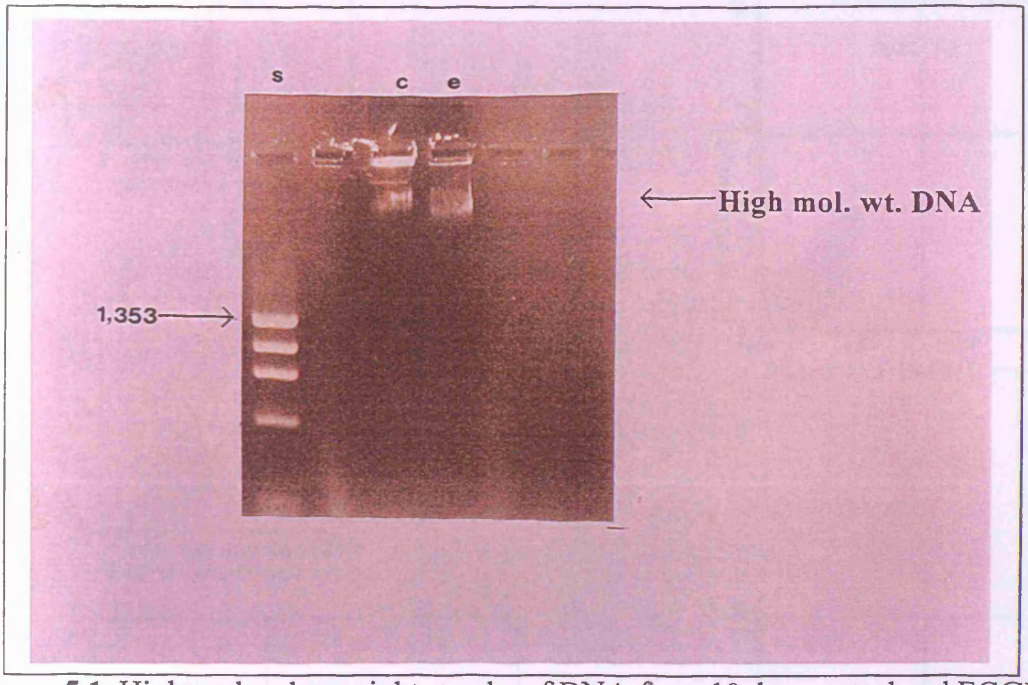



**Figure 5.1.** High molecular weight streaks of DNA from 10 day control and ECCM treated cells. The streaks appear at a much higher base pair length than the 1,353 base pair marker indicated. There is no sign of any DNA laddering normally associated with apoptotic cells.

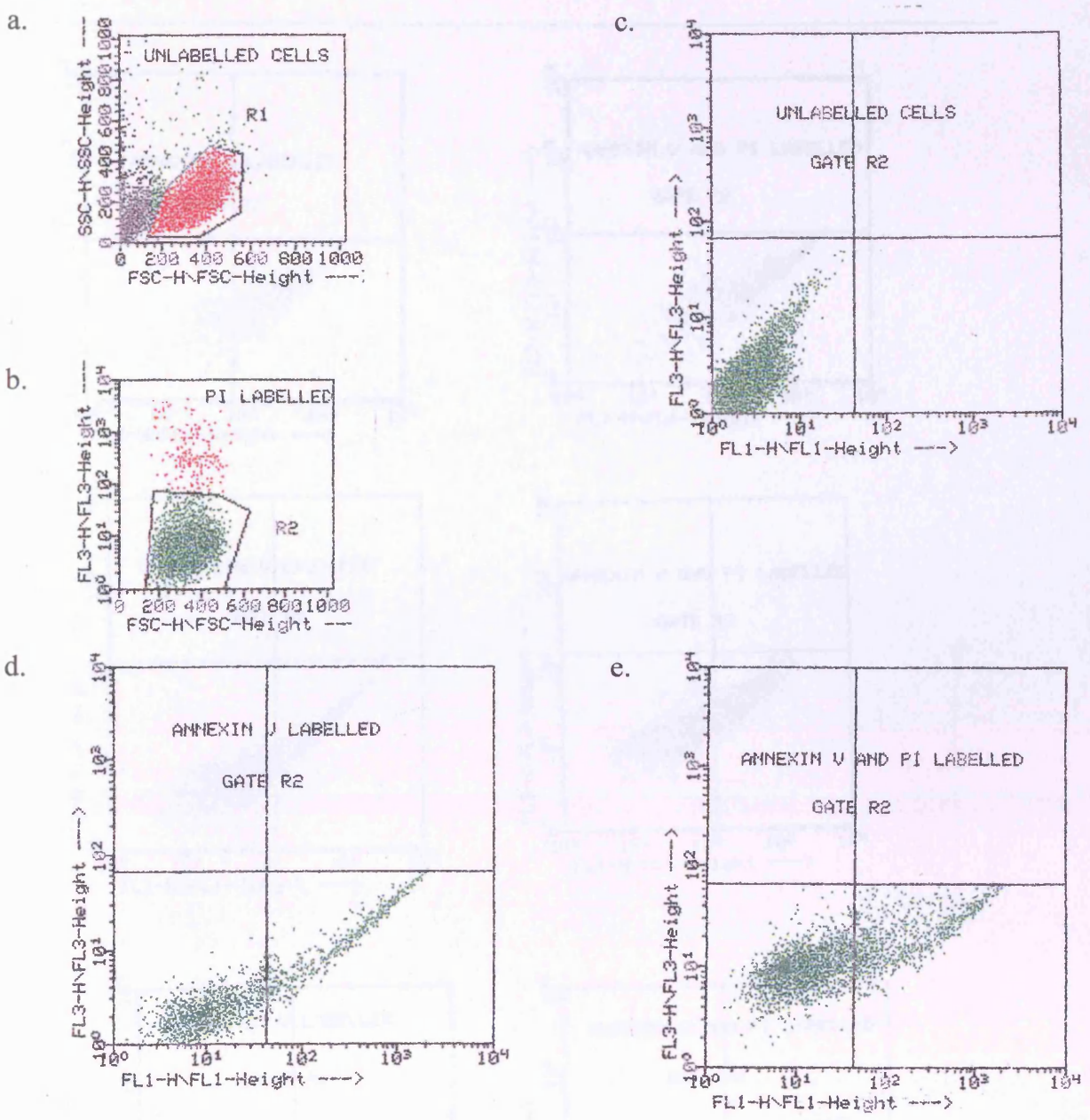



**Figure 5.2.** Results of flow cytometric analysis of freshly dissociated hypertrophic chondrocytes (a) The distinct colony, likely to be intact cells, is gated (R1) in terms of size (forward scatter height (FSC-H) against side scatter height (SSC-H)). (b) PI labelled cells from R1 are examined via FL-3 height (FL3-H) to determine cells positive for PI. Those negative for PI are gated for further examination (R2). (c) Unlabelled cells are mapped on FL1-H against FL3-H to give the maximum fluorescence without any label. Quadrants were then drawn to place all cells in the lower left quadrant i.e all cells are FL1 and FL3 negative. (d) Annexin V labelled cells from R2 were examined on FL1 against FL3, the quadrants were copied to give the percentage of cells which migrate to the right i.e. FL-1 positive, and are therefore labelled with annexin V. (e) The same examination was performed on annexin V and PI labelled cells.

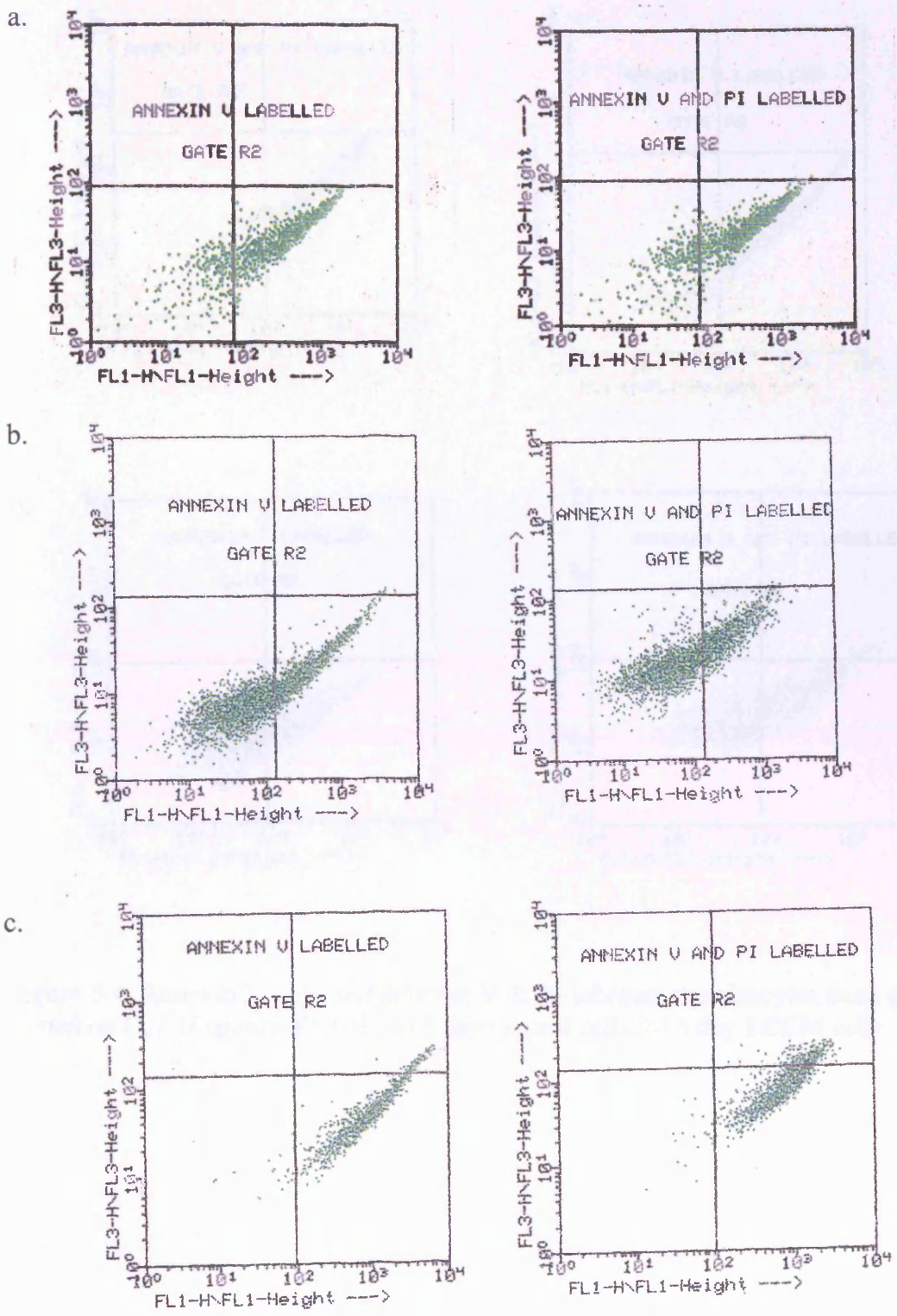



**Figure 5.3.** Annexin V only and annexin V & PI labelled chondrocytes from gate R2 plotted on FL1-H against FL3-H. (a) Staurosporin treated cells, (b) 2 day control cells, (c) 2 day ECCM treated cells.

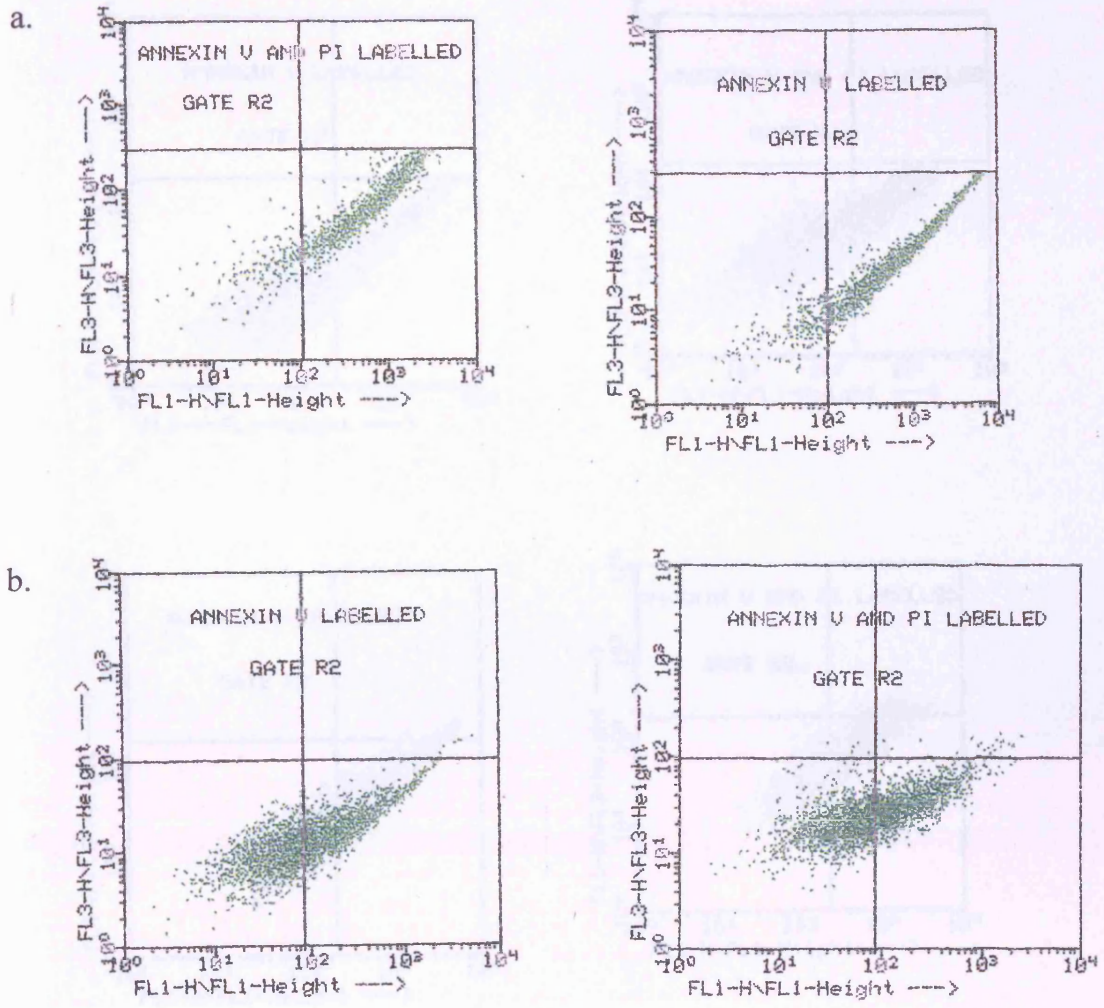



**Figure 5.4.** Annexin V only and annexin V & PI labelled chondrocytes from gate R2 plotted on FL1-H against FL3-H. (a) 5 day control cells (b) 5 day ECCM cells.

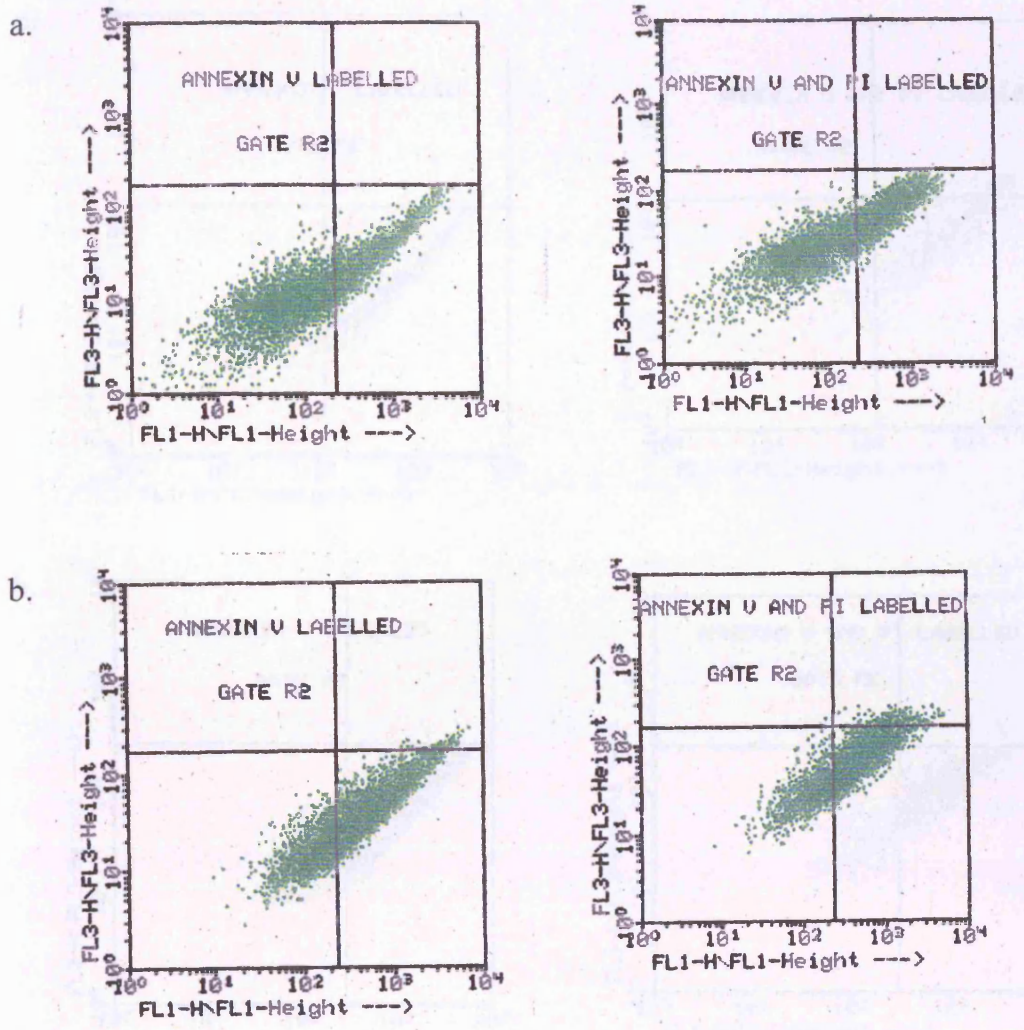



**Figure 5.5.** Annexin V only and annexin V & PI labelled chondrocytes from gate R2 plotted on FL1-H against FL3-H. (a) 7 day control cells (b) 7 day ECCM cells.

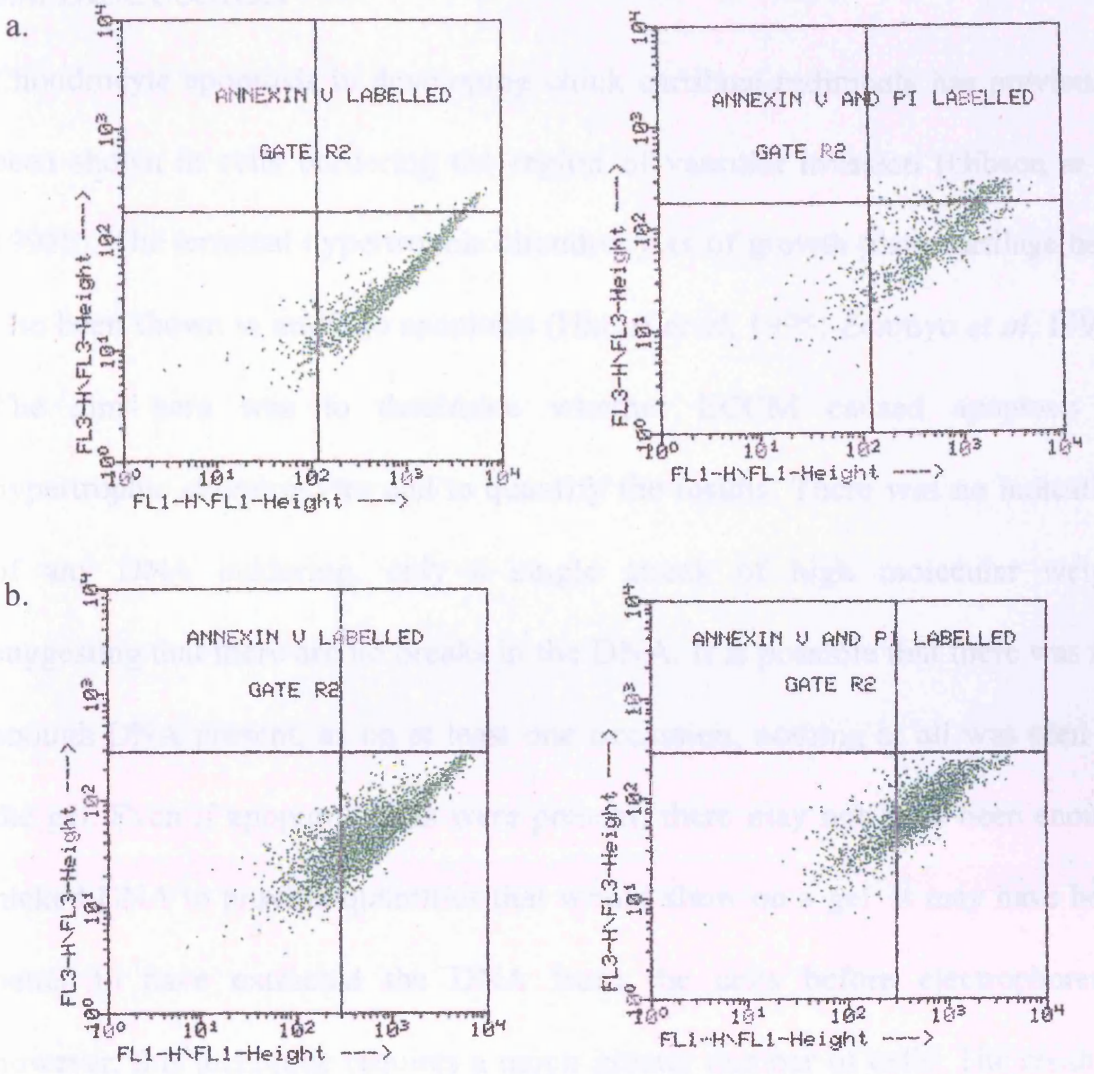



**Figure 5.6.** Annexin V only and annexin V & PI labelled chondrocytes from gate R2 plotted on FL1-H against FL3-H. (a) 10 day control cells (b) 10 day ECCM cells.

## 5.4. DISCUSSION

Chondrocyte apoptosis in developing chick cartilage rudiments has previously been shown in cells bordering the region of vascular invasion (Gibson *et al*, 1995b). The terminal hypertrophic chondrocytes of growth plate cartilage have also been shown to undergo apoptosis (Hatori *et al*, 1995; Zenmyo *et al*, 1996). The aim here was to determine whether ECCM caused apoptosis in hypertrophic chondrocytes and to quantify the results. There was no indication of any DNA laddering, only a single streak of high molecular weight suggesting that there are no breaks in the DNA. It is possible that there was not enough DNA present, as on at least one occassion, nothing at all was seen on the gel. Even if apoptotic cells were present, there may not have been enough nicked DNA to provide quantities that would show on a gel. It may have been better to have extracted the DNA from the cells before electrophoresis, however, this technique requires a much greater number of cells. The result as taken indicate that there is no apoptosis, in control or ECCM cells either during early or late degeneration.

The results of the flow cytometry are inconclusive, though the general trend, except at 10 days, was for a greater number of annexin V-FITC positive cells in the conditioned medium cultures compared to the equivalent control culture. However, a substantial amount of control cells in each culture were also annexin V-FITC positive, though showed no morphological evidence of apoptosis. Equivalent cultures have survived, on occasion, for up to 30 days.

This was an initial pilot study, which was hoped would give some indication as to whether culture in ECCM caused apoptosis or not. There are several explanations that could account for the results achieved. The most simple is that membrane damage occured during harvesting of the cells (van Engeland *et al.*, 1996). This could account for the variability between control cultures, as the different samples are unlikely to be exposed to the trypsin for identical lengths of time, the longer the trypsin exposure, the more damage is likely to occur. A second explanation for the large percentage of annexin V positive cells could be the presence of matrix vesicles. These are present within the matrix of cartilage (Anderson, 1995) and are enriched in phosphatidyl serine (Genge *et al.*, 1990). These may be released and/or disrupted during harvesting, exposing a large concentration of phosphatidyl serine, to which the annexin V binds.

One further explanation is that annexin V is the human analogue of chick anchorin CII, which is a collagen binding protein, specifically binding native collagens type II and X, and also chondrocalcin (Kirsch & Pfaffle, 1992). Type II and X collagens are the major collagenous products of hypertrophic chondrocytes (Solursh *et al.*, 1982; Kielty *et al.*, 1985; Horton & Hassell, 1986; Kirsch *et al.*, 1992; Sullivan *et al.*, 1994). Some collagen may remain attached to the surface of the chondrocytes when they are harvested despite enzymatic treatment. Staining of a cell suspension, after removal by trypsin, with by the Van Gieson technique (Bradbury & Gordon, 1990) could be attempted to determine the presence of collagen at the cell surface. If this is

the case, then annexin V will bind to these proteins, making these cells annexin V positive without actually expressing cell surface phosphatidyl serine. One method of getting round this would be to treat the cells with collagenase before the addition of annexin V, as was the case with the freshly dissociated cells.

In the light of annexin V and anchorin CII being the same molecule the use of annexin V in determining apoptosis of cartilage cells is a poor method. Even if the flow cytometric process were to be optimised for the specific cells, the fact that annexin V binds to components of the cartilage matrix means that there is still much scope for non-phosphatidyl serine specific binding of the annexin V.

# CHAPTER 6

# An *In-Vivo* Model of Human Cartilage Vascularisation

## 6.1. INTRODUCTION

During OA, the tidemark of articular cartilage is violated by blood vessels from the underlying bone (Mankin, 1974b; Jeffery, 1994b). However, this has only recently been considered to be an important factor in the disease (Brown & Weiss, 1988). The reason and role for this cartilage neovascularisation is still to be fully elucidated. For the vasculature to invade the cartilage, which is normally an avascular tissue (Hamerman, 1989; Kuettner 1992), and considered to be anti-angiogenic (Moses *et al.*, 1990; Moses, 1993) it is reasonable to assume that there must be some change within the cartilage, particularly in the ECM, as this is the physical barrier which must be penetrated.

Numerous changes have been demonstrated in terms of cartilage matrix components during OA (Mankin *et al.*, 1971; Venn & Maroudas, 1977; Christensen & Reiman, 1980; Adam & Deyl, 1983; Hoyland *et al.*, 1991; von der Mark, 1992; Aigner *et al.*, 1993a; Lafeber *et al.*, 1993) and it is possible that some of these changes are related to invasion by the vasculature.

In an attempt to determine whether human OA cartilage retained its anti-angiogenic properties, slices of OA cartilage from the knees of patients undergoing total knee replacement were grafted onto the chick CAM and assessed for vascular invasion.

Various histochemical stains were employed to examine any changes in proteoglycan content, and immunostaining for collagen types I, II, III and X was

performed to examine any changes in collagen expression in CAM grafted and non-CAM grafted OA and healthy cartilage.

## 6.2. MATERIALS AND METHODS

*Human Tissue Harvesting*

Human OA cartilage was obtained direct from theatre after total knee replacement (TKR) from 5 male and 4 female patients between the age of 58 and 79. Healthy human cartilage was obtained from the ankle joint of a female aged 67 undergoing above knee amputation and from 3 femoral heads of 3 females aged 68, 71 and 77, undergoing hip replacement for fractured neck of femur.

Cartilage was taken direct from theatre and harvesting of tissue was performed aseptically in a class 2 microbiological safety cabinet. The femoral condyles of the OA knees were cut into thin strips with a pair of bone cutters. The cartilage, taken from areas of relatively healthy looking cartilage that was at least 3mm in depth, was then carefully cut away from the subchondral bone at the bone cartilage interface with a scalpel to maintain the full thickness of the cartilage. Slices of cartilage 1mm thick were taken for use in CAM grafting experiments. Slices of ankle and femoral head cartilage were cut off with a scalpel down to the underlying bone to maintain the full thickness of the cartilage. Slices of 1mm thickness were again taken for use in CAM grafting experiments.

*CAM Grafts*

Fertilized White Leghorn chicken eggs were obtained from a registered supplier

and incubated until 10 days of age. The 1mm thick slices of cartilage were cut in half. One half was laid flat onto the CAM of 10 day old chick embryos, 3 pieces per CAM in each of 24 eggs, and incubated at 37°C for 5 days. After the incubation period, the grafts were removed. the excess CAM tissue removed, and the grafts prepared for either wax histology or cryosectioning. The second, ungrafted, half was prepared directly for similar treatment.

*Histology and Histochemistry*

5 OA patient sets of grafted and ungrafted cartilage and the ankle cartilage were fixed in 10% neutral buffered formalin (Appendix II) for up to a week before processing for wax histology and histochemistry. The grafted cartilage was trimmed of excess CAM tissue before processing.

7μm sections were cut on a sledge microtome and mounted on 3-aminopropyltriethoxy-silane (silane, Sigma) coated slides. Every fourth section was stained with haemotoxylin and eosin for general morphology. A number of other sections were stained with safranin-O (Appendix II) or toluidine blue (BDH) (1% aqueous) for proteoglycans or with alcian blue at critical electrolyte concentrations (CEC) of 0.06M or 0.7M $MgCl_2$ (Appendix II) for non-specific and cartilage specific GAG staining respectively (Scott & Dorling, 1965).

*Cryosectioning*

The remaining 4 OA patient sets and the femoral head CAM grafted cartilage was excised and trimmed of excess CAM tissue before embedding in Cryo-M-

Bed (Bright) on a piece of cork and snap frozen in liquid nitrogen. Non-grafted cartilage was snap frozen in an identical manner. Samples were stored at -80°C until ready for sectioning. Sections were cut on a Bright cryostat model OTF with a blade specifically designed for skeletal tissue. 7μm sections were cut and mounted on clean silane (Sigma) coated slides, before air-drying at room temperature for 60 minutes. The sections were then fixed in analytical grade acetone (Fisher) for 10 minutes and air-dried before wrapping in foil and storage at -20°C until ready for staining.

*Immunostaining*

Sections were brought to room temperature then unwrapped and hydrated in TBS. After treatment with testicular hyaluronidase (Type I-S, Sigma) 1mg/ml in acetate buffer (Appendix II) for 1 hour, the sections were rinsed in TBS and flooded with normal rabbit serum (Serotec) diluted 1:5 with TBS for 10 minutes. Serum was then removed but not rinsed, before addition of primary antibody. The primary antibodies used were goat anti-human type I collagen (Southern Biotechnologies), mouse anti-type II collagen (CIICI, DSHB), goat anti-human type III collagen (Southern Biotechnologies) and mouse anti-human type X collagen (MB6, A kind gift from Dr. A. Kwan, University of Wales, Cardiff). Dilutions were made as recommended, 1:20 for type I and III (manufacturers recommendations), 1:5 for type II (G. Riley, Addenbrookes Hospital, Cambridge, personal communication) and 1:100 for type X. All dilutions were made in TBS. Slides were incubated for 1 hour at room

temperature, then rinsed in TBS before addition of appropriate secondary antibody, either rabbit anti-goat peroxidase conjugated secondary antibody, or rabbit anti-mouse biotin conjugated antibody respectively (Dako) at 1:200 dilution in TBS for 1 hour at room temperature. Peroxidase labelled sections were rinsed and incubated in DAB/$H_2O_2$ solution (Sigma Fast DAB Tablets, Sigma) for 3-5 minutes, rinsed again before counterstaining in Mayers haemotoxylin (Appendix II), dehydrating and mounting in DPX (BDH). Biotin labelled sections were rinsed before addition of ABComplex (Dako) for 20-30 minutes, rinsed again then incubated with DAB (Sigma) for 3-5 minutes. Slides were counterstained with haemotoxylin then dehydrated before mounting in DPX mountant (BDH) and visualised on an Olympus BH2 light microscope.

## 6.3. RESULTS

All OA tissue generally had a jaundiced appearance and varied in thickness from approx. 1mm to 3-4mm, only the thicker pieces were used for grafting. It had lost the shiny hyaline look associated with normal cartilage (Mankin, 1974a; Howell *et al.*, 1976; Hamerman, 1989). During incubation on the CAM, almost all pieces of cartilage became at least partially enveloped in CAM tissue containing blood vessels (Figure 6.1). However, there was no leakage of blood, in contrast to chick embryonic long bone rudiment cartilage (see Figure 2.1).

*Histology and Histochemistry*

CAM grafted sections stained with H&E revealed that all OA cartilage became invaded by CAM derived cells to varying extents. Invasive tissue appeared as either groups of single cells, lines of cells or occasionally as a proper lumen containing erythrocytes (Figure 6.2). None of the healthy cartilage, despite becoming enveloped in CAM tissue containing blood vessels, showed any vascular invasion. Invasion occurred through the deep layers and possibly from the side of the deep layers of the sections. Invasion never occurred through the surface. The majority of the invasive cells were morphologically fibroblastic, however, a small proportion of the cells were more rounded, stained darkly and contained darkly staining granules (Figure 6.3). Chondrocytes, appeared healthy, many had proliferated to form clusters of cells known as chondrones (Figure 6.4) (Archer, 1994), which also appear healthy. OA cartilage, fixed and

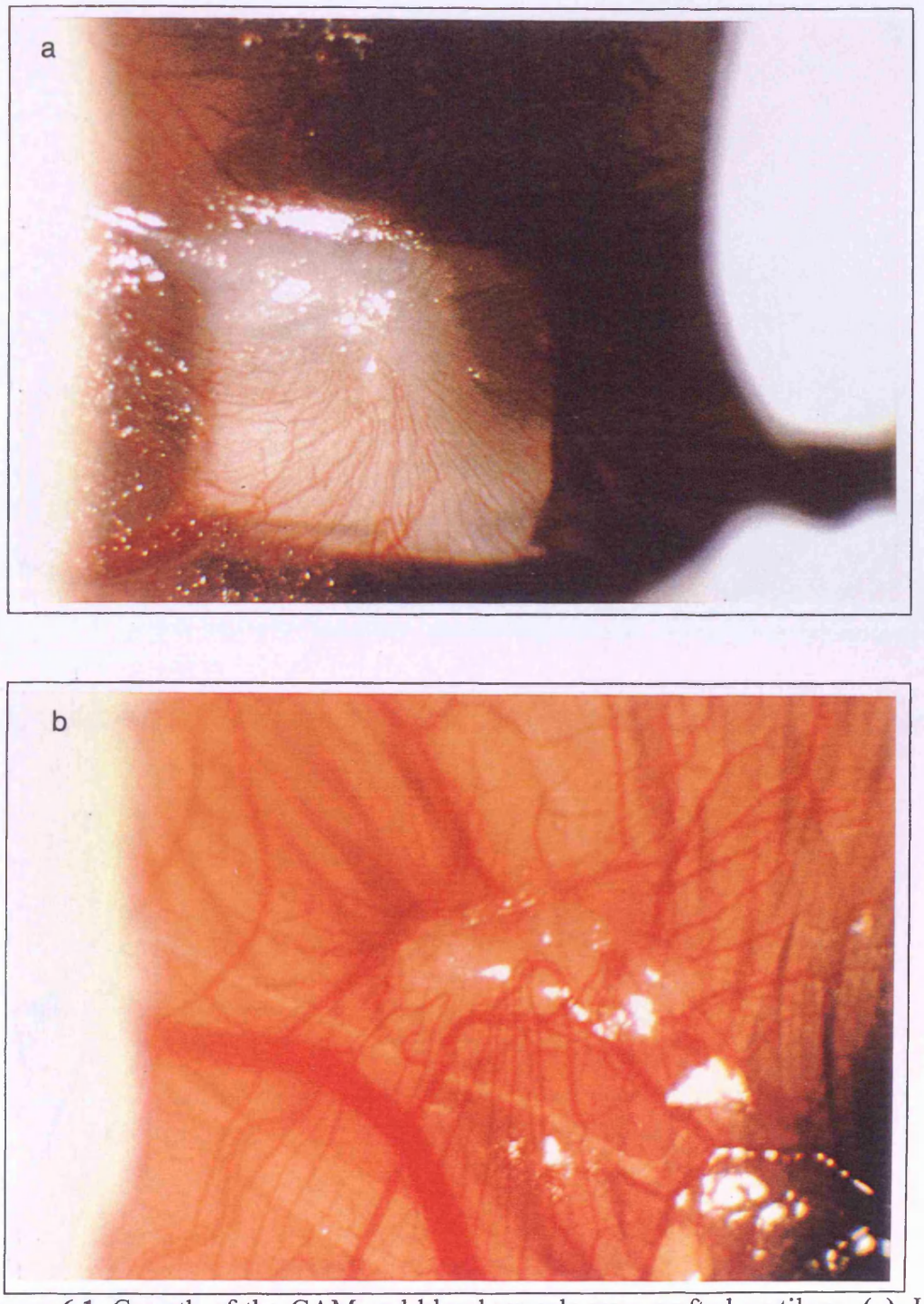



**Figure 6.1.** Growth of the CAM and blood vessels over grafted cartilage. **(a).** Healthy cartilage from the ankle joint of an above knee amputee. (x 30) **(b).** OA cartilage from the femoral condyle of a patient undergoing total knee replacement. (x 20).

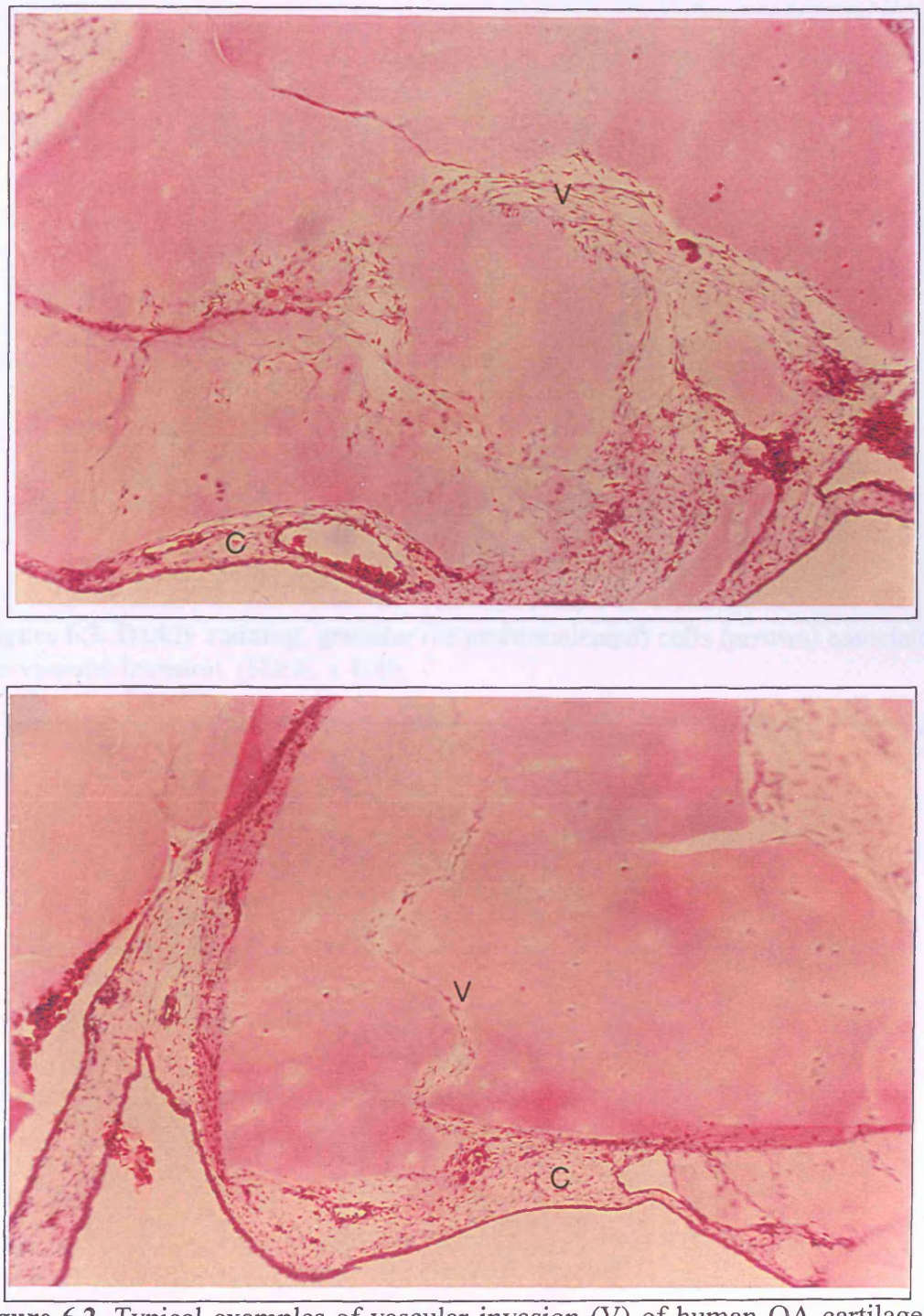



**Figure 6.2.** Typical examples of vascular invasion (V) of human OA cartilage when grafted onto the chick CAM (C). (H&E, x 100)

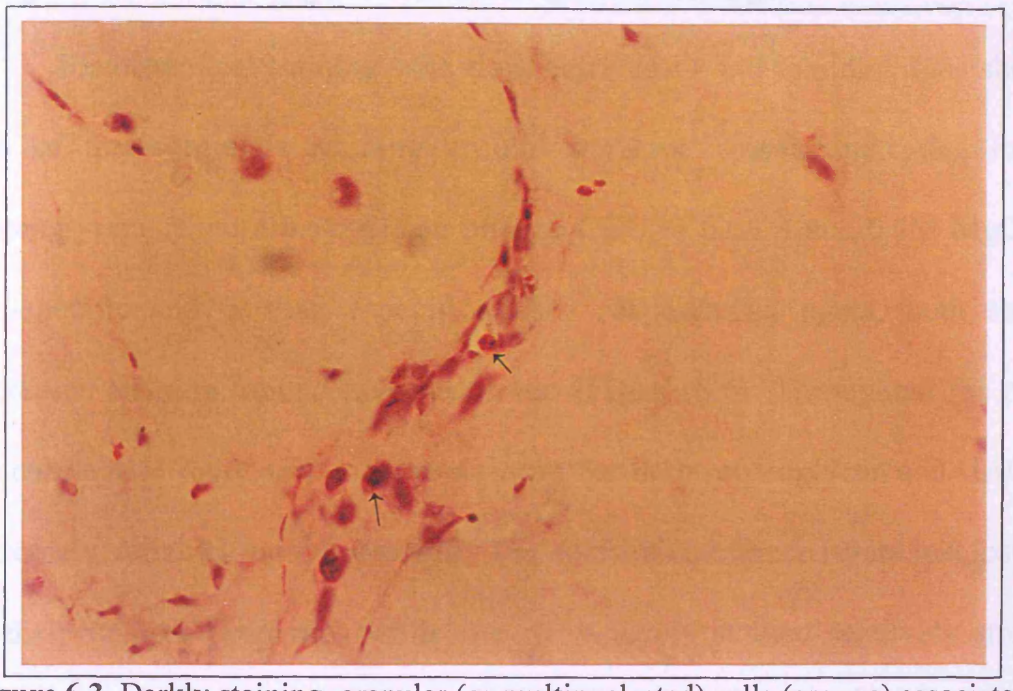


**Figure 6.3.** Darkly staining, granular (or multinucleated) cells (arrows) associated with the vascular invasion. (H&E, x 400).

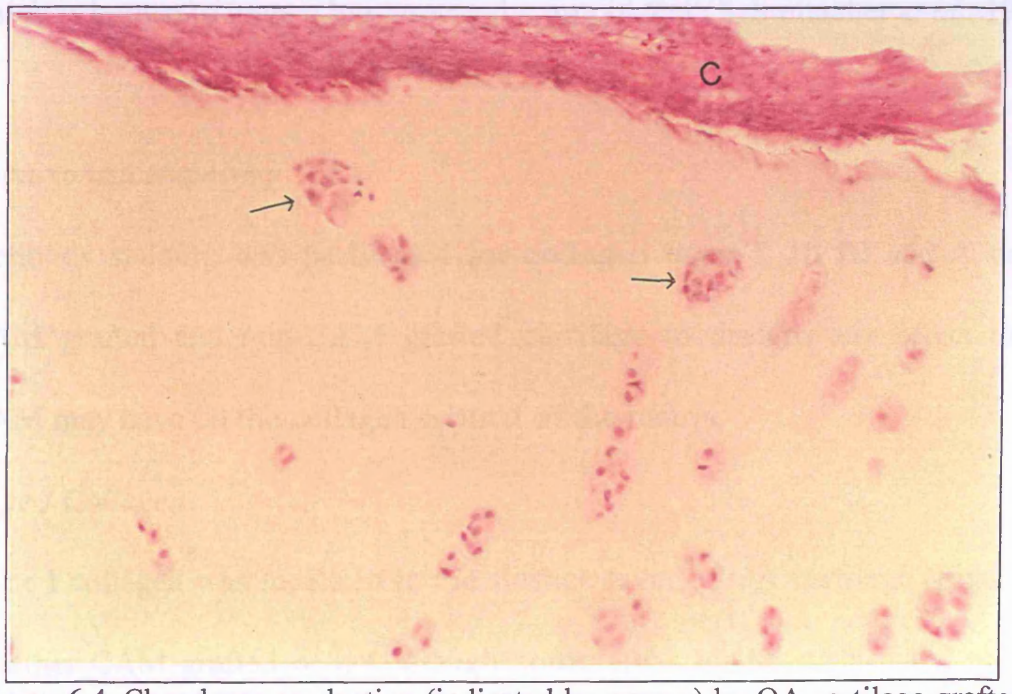



**Figure 6.4.** Chondrone production (indicated by arrows) by OA cartilage grafted onto the CAM (C). (H&E, x 200).

processed with no culture on the CAM. also showed chondrone production, though this occured to a greater extent in grafted cartilage.

Histochemical staining with both safranin-O and toluidine blue shows a loss of metachromatic staining around invasive vessels indicating loss of proteoglycans (Figure 6.5). Alcian blue at CEC of 0.06M and 0.7M $MgCl_2$ for non-specific and cartilage specific GAG respectively, again, both show a decreased staining around invasive tissue (Figure 6.6). Throughout the rest of the cartilage in these sections, the staining for both proteoglycan and GAG was extremely variable, though generally the surface and upper layers had lost a lot of their staining properties while the deep layers stained relatively strongly. Healthy cartilage stained uniformly throughout except the very surface layer, which is known to have a low proteoglycan content (Schumacher *et al.*, 1994)

*Immunohistochemistry*

Antibody staining was performed for collagen types I, II, III and X on both CAM grafted and non-CAM grafted cartilage to discern any effect that the CAM may have on the collagen content of the matrix.

*Type I Collagen:*

Type I collagen was localised to the surface layer of OA cartilage (Figure 6.7), whether CAM grafted or not, though some areas of the surface did not stain, most likely due to previous erosion during the disease process. In CAM grafted cartilage the chondrocytes at the surface stained faintly, but those in the deeper

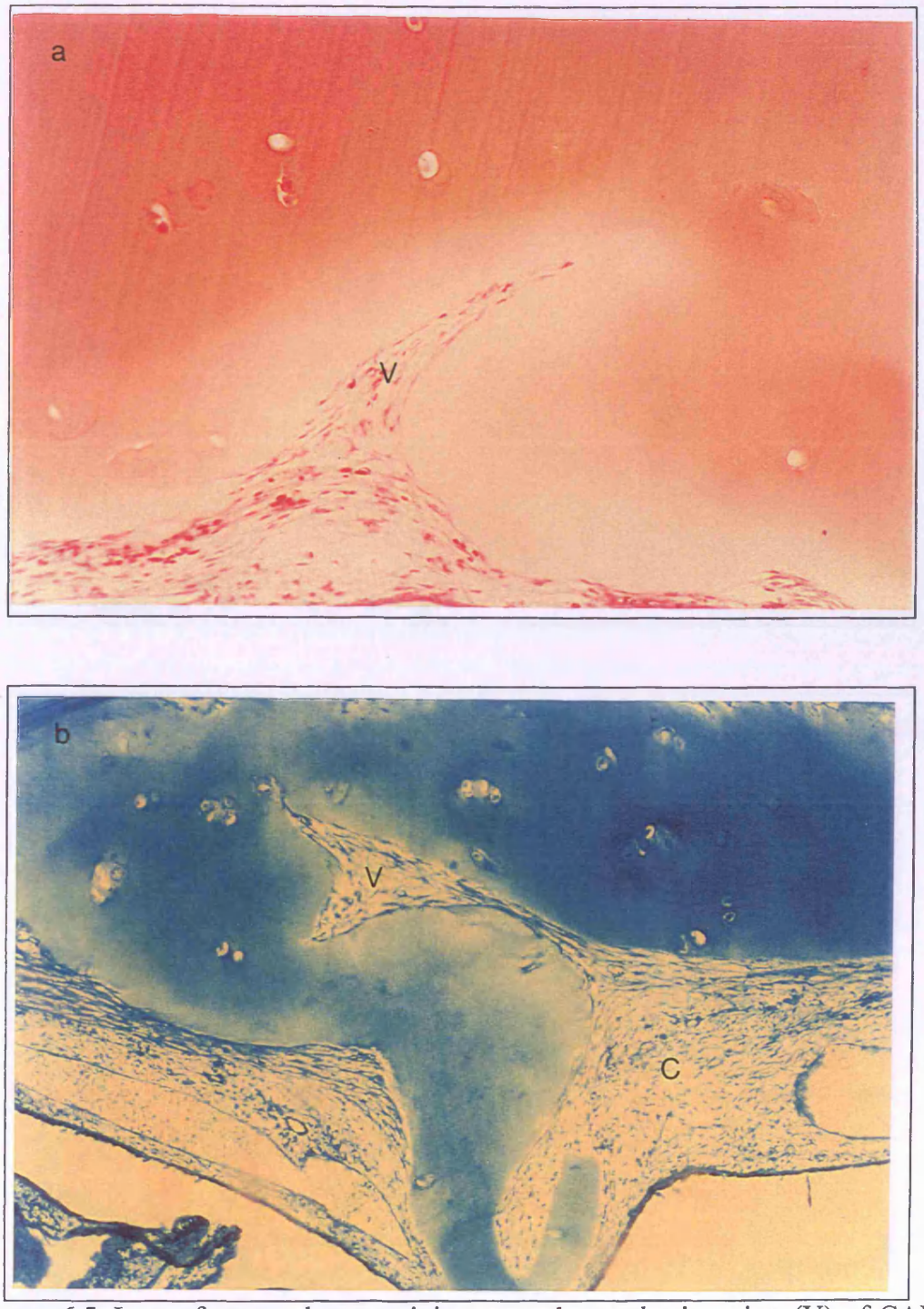
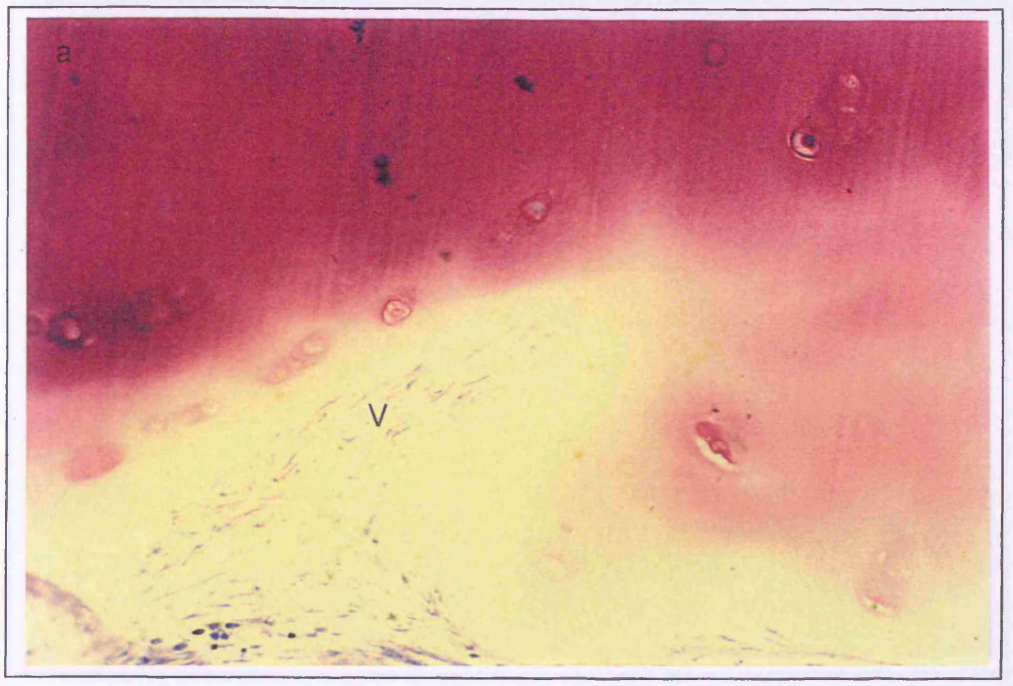



**Figure 6.5.** Loss of proteoglycan staining around vascular invasion (V) of CAM (C) grafted OA cartilage. **(a).** Safranin-O (x 400). **(b).** Toluidine blue (x 200).



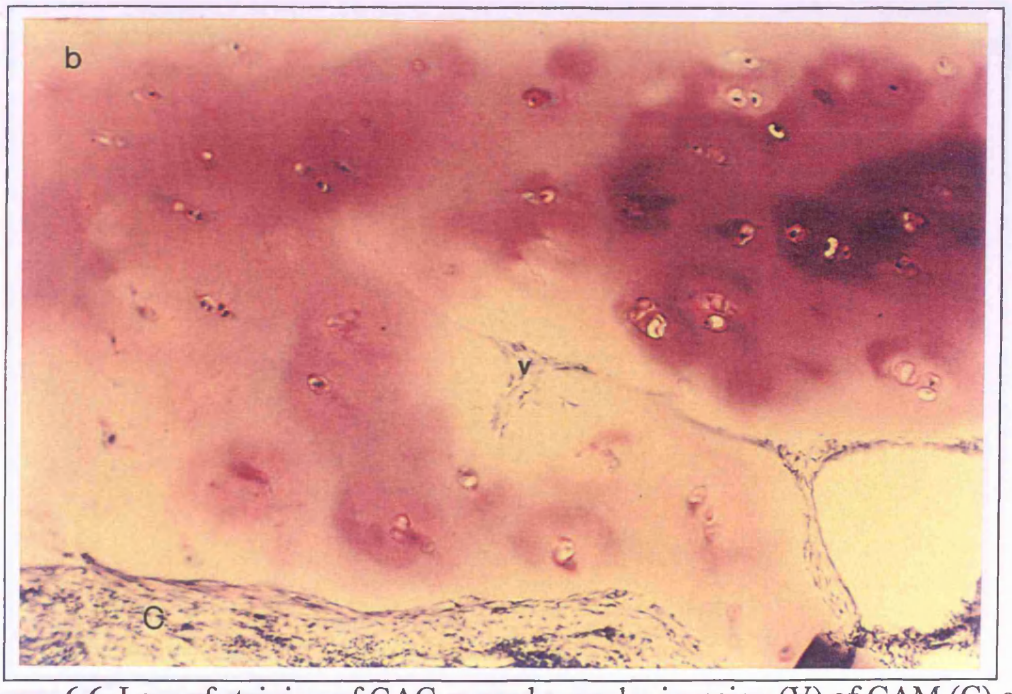




**Figure 6.6.** Loss of staining of GAG around vascular invasion (V) of CAM (C) grafted OA cartilage. **(a).** Alcian blue CEC 0.06M $MgCl_2$ (x 200). **(b).** Alcian blue CEC 0.7M $MgCl_2$ (x 400).

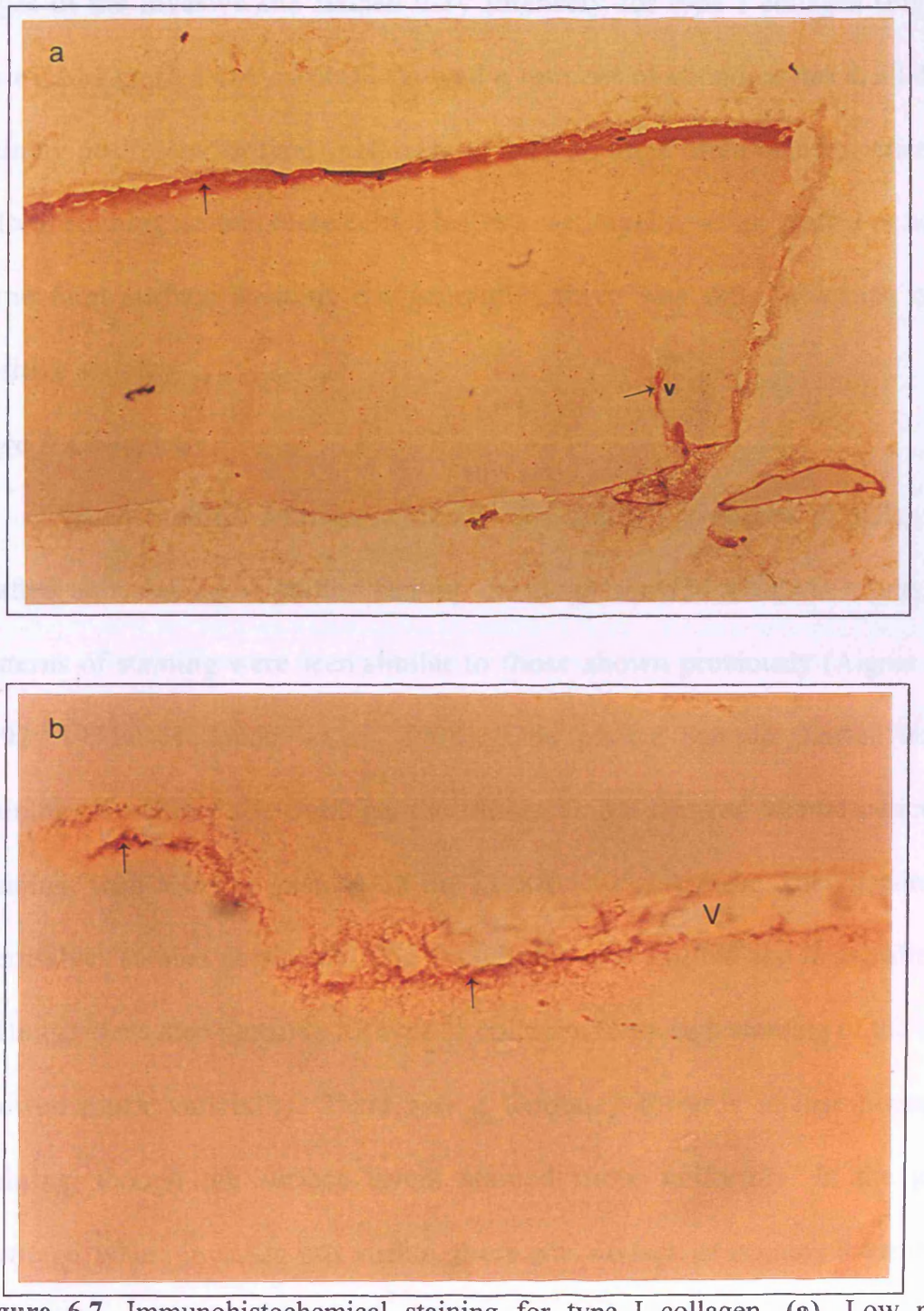



**Figure 6.7.** Immunohistochemical staining for type I collagen. **(a).** Low power micrograph showing surface staining and staining of the matrix around vascular ingrowth (v). Arrows indicate positive staining (x 40). **(b).** High power micrograph showing positive matrix staining for type I collagen (arrows) around the site of vascular invasion (V). (x 400).

layers were virtually all negative. When vascular invasion was clearly seen, the edges of the invasive site stained very intensely for type I collagen (Fig 6.7). Non-CAM grafted OA cartilage showed a number of chondrocytes at all depths staining positively for type I collagen, as well as there often being a pericellular halo of staining around these cells. Healthy cartilage, whether grafted or not had some faint surface staining, but generally, there was little indication of any positive staining.

*Type II Collagen:*

When stained with an antibody for type II collagen (CIICI), CAM grafted and non-CAM grafted healthy cartilage showed identical results. Two patterns of staining were seen similar to those shown previously (Aigner *et al.*, 1992; 1993a; Hollander *et al.*, 1994). One patient sample showed uniform staining throughout the cartilage, the other sample showed intense pericellular staining, with a lower intensity in the interterritorial matrix. The chondrocytes themselves stained negatively. The chondrocytes in grafted and non-grafted OA cartilage were also negative for type II collagen, although staining of the matrix showed much variability. There was a tendency towards intense pericellular staining, though the surface layers stained more uniformly. In the grafted cartilage, where invasion was visible, there was no lack of staining intensity.

*Type III Collagen:*

The surface layers of almost all samples of healthy and OA cartilage types stained lightly and uniformly for type III collagen, while the deep chondrocytes showed intense pericellular staining. The chondrocytes

themselves did not stain. In these deeper layers, there was no indication above background staining in the inter-territorial matrix (Figure 6.8). In sections of OA CAM grafted cartilage which showed vascular invasion, there was no indication of type III collagen staining around the invasion site.

*Type X Collagen:*

Unlike the staining for collagen types I, II and III where there was very little variation in the staining properties between different samples, staining for type X collagen showed a number of patterns which have been seen previously (Aigner *et al.*, 1993b). All samples showed positive staining of the chondrocytes to varying extents. Non-grafted healthy cartilage generally showed a positive result in all chondrocytes throughout the sections, though the matrix showed no evidence of positive staining except in the occasional section where there was faint staining at the extreme surface. In healthy grafted cartilage, no indication of surface staining could be seen, but there were signs of pericellular staining for type X collagen in some of the chondrocytes both near the surface and in the deeper layers in many sections. However, a few sections did not show this pericellular staining. Non-grafted OA cartilage stained very similarly to the healthy cartilage though occasionally, the surface showed some positive staining, and some deep cells showed some faint pericellular staining. Grafted OA cartilage showed general positive staining of all chondrocytes and some indications in a number of sections of positive surface staining. The matrix showed no indication of staining except around the invading vasculature where the edge of the matrix around the invasion stained positively (Figure 6.9). This

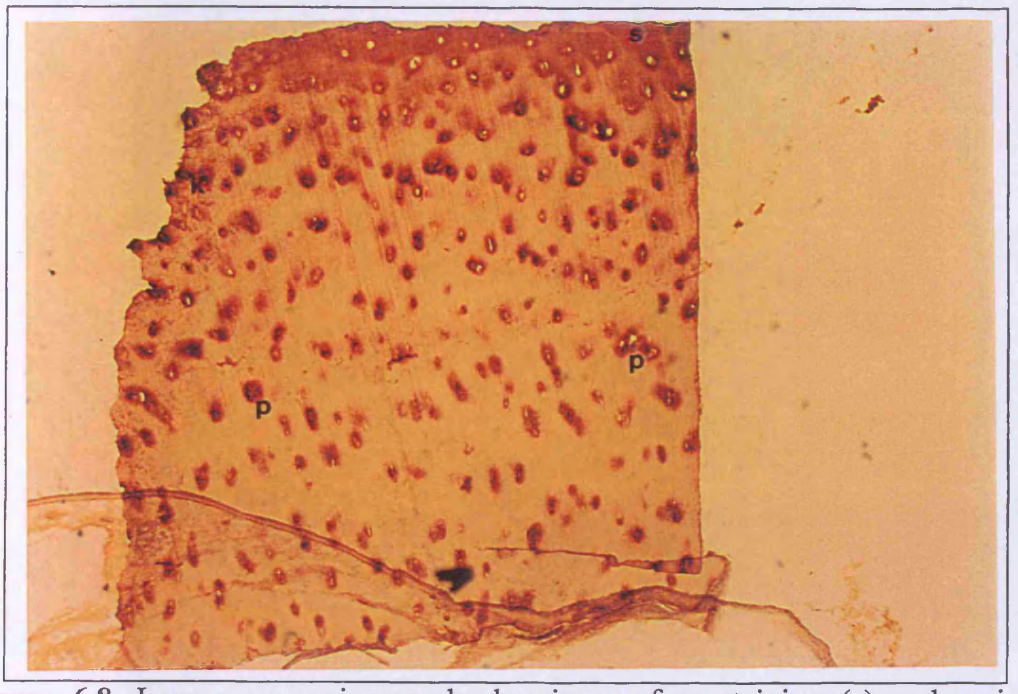



**Figure 6.8.** Low power micrograph showing surface staining (s) and pericellular staining (p) for type III collagen in CAM grafted OA cartilage. (x 40).

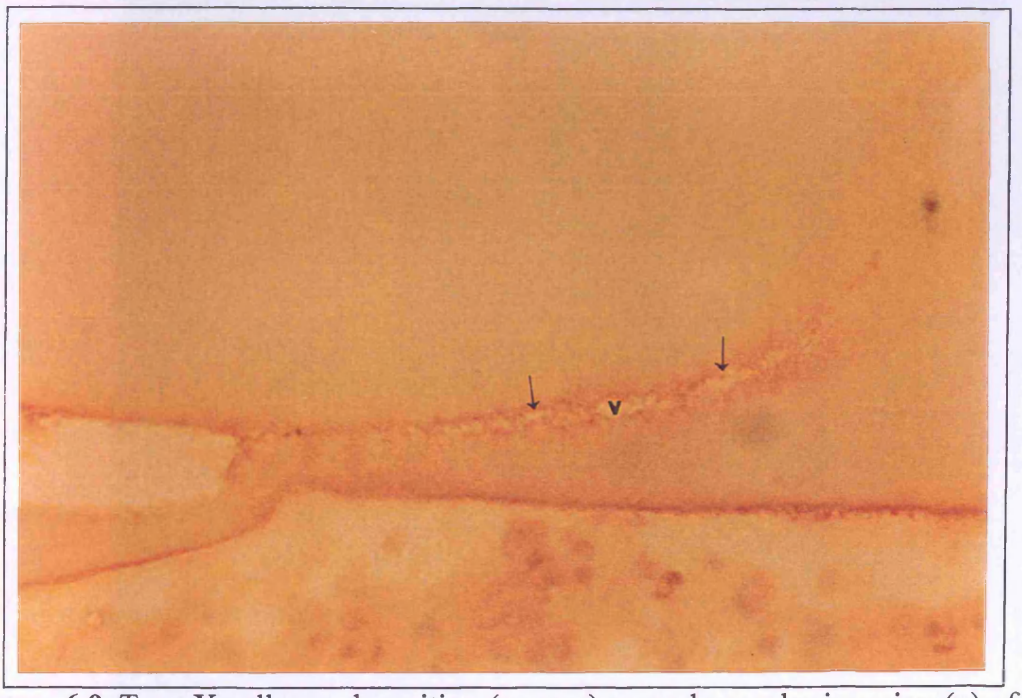



**Figure 6.9.** Type X collagen deposition (arrows) around vascular invasion (v) of CAM grafted OA cartilage. (x 400).

rim of positive staining was not as intense and did not penetrate as deeply into the matrix as the type I collagen staining.

## 6.4. DISCUSSION

It has been demonstrated here that human OA articular cartilage loses its ability to remain avascular when placed into the *in-vivo* model of vascularisation, that of the chick CAM. Changes in the ECM components were examined by both histochemical and immunohistochemical staining. Most notable is the observation that vascular invasion was always associated with a localised loss of proteoglycan/GAG staining within the cartilage ECM. CAM grafting did not have any noticeable effect on the rest of the matrix. This, however, may not be such a surprise. As incubation time is relatively short and it is known that cartilage ECM components have a slow turnover time within cartilage matrix (Mankin, 1974b; Lippiello *et al.*, 1977), there is unlikely to be a significant cartilage mediated change over such a short period of time.

The results of the study are similar to previous studies that have examined types I, II, III and X collagen and proteoglycan/GAG (Mankin *et al.*, 1971; Venn & Maroudas, 1977; Christensen & Reiman, 1980; Adam & Deyl, 1983; Hoyland *et al.*, 1991; von der Mark, 1992; Aigner *et al.*, 1993a; Lafeber *et al.*, 1993). Types I and III are often found confined to the surface layer of normal cartilage, while type III in OA cartilage is also located pericellularly (Adam & Deyl, 1983; Aigner *et al.*, 1993a; Nerlich *et al.*, 1993). However, healthy cartilage stained here for type III also showed pericellular staining for this collagen. Type II collagen showed general uniform staining throughout both control and OA cartilage sections, as would be expected, and has been shown

previously (Aigner *et al.*, 1993a; Nerlich *et al.*, 1993). Aigner *et al.*, (1992) however, also showed some irregular staining of type II collagen in OA cartilage, via enhanced pericellular deposition and diminished inter-territorial staining.

Type X collagen stained to varying degrees in both healthy and OA cartilage at the surface layers, and in the pericellular region of a number of the chondrocytes and also intra-cellularly in the majority of chondrocytes. Healthy cartilage has previously shown little or no type X collagen staining (Kirsch *et al.*, 1992; Aigner *et al.*, 1993b), while OA cartilage has been shown to stain for type X collagen both intra-cellularly and pericelluarly, most prominently around hypertrophic cells and proliferating clusters of cells in the upper and middle zones of the cartilage (von der Mark *et al.*. 1992; Aigner *et al.*, 1993b).

The most prominent feature of the staining of this cartilage was of the invasive vessels seen in CAM grafted OA cartilage. The rim of matrix around the invasion stained positively for both type I collagen and type X collagen. Type I collagen is associated with dedifferentiated chondrocytes (Mayne *et al.*, 1976; Kirsch *et al.*, 1992; Bonaventure *et al.*, 1994). It is also a component of blood vessels and is known to be up regulated in endothelial cells undergoing angiogenesis (Rooney *et al.*, 1993). Type X has been hypothesised to be a factor in the calcification/mineralisation and vascularisation of cartilage (Schmid *et al.*, 1990; Iyama *et al.*, 1991; Rooney & Kumar, 1993; Wallis, 1993). Another important observation is that the monoclonal antibody to type X collagen is known to cross react with type VIII collagen (P. Rooney, personal

communication), which has also been associated with the vasculature (Kittelberger *et al.*, 1989). The source of the type I collagen could either be cartilage or vasculature derived as the anti-type I antibody is a polyclonal shown to cross react with both human and chick type I. The anti-type X antibody is specific for human type X collagen therefore the type X (or possibly type VIII) laid down in the matrix must be cartilage derived.

OA cartilage has been shown previously to have great variation in its proteoglycan staining properties (Mankin *et al.*, 1971; Christensen & Reimann, 1980), and the results presented here confirm this. However, as with the collagen staining, there is a specific change in matrix staining around invasive vessels.

OA cartilage has never previously been grafted onto the CAM, therefore this work provides a novel model for the study of active vascularisation of human OA cartilage. The process of vascularisation of the human OA cartilage on the CAM occurs rapidly. The study of this process can therefore be enhanced and the model may aid in elucidating the reasons as to why the process occurs at all.

In conclusion, human OA cartilage loses its avascular state when placed onto the chick CAM. There is a loss of non-specific and cartilage GAG and proteoglycans around the invasion site, and laying down of type I and type X collagen along the rim of vascular, invasion which may have a role in the process. There is no apparent change in type II collagen.

# CHAPTER 7

# Discussion

an established angiogenic assay (Nguyen *et al.*, 1994).

The stage at which chick embryo cartilage rudiments become invaded *in-ovo* has been well documented (Fell, 1925; Pechak *et al.*, 1986a & b). This data has been utilised here to investigate the temporal and spatial characteristics of developing cartilage vascularisation. Grafting of intact cartilage long bone rudiments and individual cellular zones onto the CAM has demonstrated that under normal conditions, vascularisation occurs at exactly the same time and place as *in-ovo*. This sequence can be influenced by brief enzymatic treatment, indicating a role for the ECM in regulating vascularisation. In addition, removal of the vascularised periosteum prevented vascular invasion, suggesting a governing role for this tissue.

These data suggest that cartilage vascularisation may be regulated by a combination of cells and factors from the periosteum, together with the specific constitution of chondrocyte ECM. Since only a small region of the central hypertrophic zone becomes newly vascularised at any given time, this implies that the vascularisation signal(s) can be very localised. The signal(s) may emerge in the centre and gradually migrate to the periphery of the zone as the hypertrophic chondrocytes become eroded.

Regardless of the stage at which rudiments were removed, time in organ culture, or the exposure of hypertrophic cartilage by dissecting away the flattened and rounded cell zones, the invasion always occurs at a central point. It is essential now to discern if and how the chondrocytes are involved in this

process. For instance whether a signal from these specific chondrocytes triggers the periosteal vasculature to initiate and subsequently invade the cartilage (Alini *et al.*, 1996). Interaction between chondrocytes and the perichondrium have been shown in the regulation of the Indian hedgehog gene (Vortkamp *et al.*, 1996). One possible test for an interaction between hypertrophic cells and the perichondrium/periosteum, *in-vitro*, might be to take perichondrium from pre-vascularised hypertrophic zones i.e. stage 34 or earlier. These could then be grafted onto BAEC monolayers to determine if tubules form. Likewise, the periosteum free cartilage could also be tested in a similar way, though assays of this cartilage on the CAM have already shown that no invasion occurs. However, the possibility does exist that the whole process is mediated by the periosteum and the results presented here do suggest this.

Breakdown of matrix components almost certainly plays a role in the triggering of the invasive process. Cartilage ECM consists primarily of collagen molecules and proteoglycan aggregates containing a hyaluronan backbone. Enzymatic treatment of cellular zones, with hyaluronidase, prior to culture on the CAM induced a more rapid vascularisation but in a similar manner to the normal vascularisation pattern. In contrast, treatment with collagenase induced widespread invasion and erosion, suggesting that hyaluronan degradation may be induced and is the more localised regulation during cartilage vascularisation. Once vascularisation has begun, the main collagenous network is possibly then degraded to allow invasion throughout the rudiment. This, however, raises a

further question as to what causes the initial breakdown of the matrix components, could it be self degeneration by specific chondrocytes at the central point that encourages the growth of vasculature into the cartilage. It may be important to note that breakdown products of hyaluronic acid are known to be angiogenic (Rooney & Kumar, 1993) and a similar role for this process has been suggested in tumour growth and metastasis (Rooney *et al.*, 1995; Rooney *et al.*, 1997) . Or alternatively, is the cartilage breakdown initiated by enzymes from the vasculature?. Another interesting question that arises from this is, what prevents the breakdown of the cartilage that remains behind as the growth plate and articular cartilage?.

*In-vitro* work with chick hypertrophic chondrocytes provides further evidence to Gibson's notion that the vasculature plays a central role in the degeneration and removal of developing cartilage (Gibson *et al.*, 1995b). The results indicate that a programme of morphological change, degeneration and death is initiated within the first 12 hours of exposure to ECCM factors. This cannot be halted, even by removal of the ECCM and replacement with fresh medium. However, some cultures did briefly return to a chondrocytic morphology, even when treated continually with ECCM. Chondrocytes that cannot change morphology due to maintenance of a rounded morphology (i.e. cultured over agarose or cultured as high density micromasses) are a great deal more sensitive to the degeneration, which may be linked to their rounded morphology and hence the

chondrocyte phenotype (Archer *et al.*, 1990). The ambiguity of the effect, the results from diluting the ECCM and the results from co-cultures of chondrocyte micromasses with different numbers of BAEC micromasses emphasise a need for quantitation of the factor(s) responsible. To achieve this, the factor(s) must first be identified.

Much work has been done on the effects of cytokines and growth factors on cartilage (Martell-Pelletier *et al.*, 1991; McCollum *et al.*, 1991; Pujol *et al.*, 1991; Chandrasekhar *et al.*, 1993; Seid *et al.*, 1993; Shingu *et al.*, 1993; van Beuningen *et al.*, 1993) and there are several obvious possibilities as to the identity of the degradatory factor(s). IL-1, TNF-α and IL-6 are commonly held mediators of connective tissue destruction (Pujol *et al.*, 1991; Pelletier *et al.*, 1993), though IL-6 has been shown not to have a detrimental effect on cartilage (Shingu *et al.*, 1993; Gunther *et al.*, 1994; Lotz *et al.*, 1995). Much of the work has focused on IL-1 and much is known about its effect on cartilage (Martell-Pelletier *et al.*, 1991; McCollum *et al.*, 1991, Pujol *et al.*, 1991; Chandrasekhar *et al.*, 1993; Pelletier *et al.*, 1993; Seid *et al.*, 1993; Lotz *et al.*, 1995), including suppression of cartilage collagens i.e. type II, and proteoglycans, while promoting the expression of fibroblastic collagen types i.e. type I, promotion of proteolytic degradation of cartilage matrix molecules and inhibition of chondrocyte proliferation. IL-1 has also been noted to promote the proliferation of fibroblasts (Tiku *et al.*, 1992) which is one of the major events seen during the culture of chick hypertrophic chondrocytes in ECCM. Articular

chondrocytes are able to express IL-1 receptors (McCollum *et al.*, 1991) and also IL-1 itself (Tiku *et al.*, 1992) and it has been shown that cartilage is able to initiate its own destruction (Kistler *et al.*, 1991; Tiku *et al.*, 1992). One possibility is that culture of chondrocytes in ECCM causes the chondrocytes to commence their own self destruct programme as seen after treatment with retinoic acid (Kistler *et al.*, 1991). As an example, the ECCM causes the chondrocytes to produce IL-1, which subsequently binds to the IL-1 receptors of the chondrocytes causing the effects described above.

Though there is no particular evidence to suggest that IL-1 is involved however, it is a good candidate for at least some of the processes described. The possibility also exists that the effect is due to a novel factor. It is most likely, however, that there is some interaction between numerous factors, as the maintenance of cartilage homeostasis requires interaction between many cytokines and growth factors (Pelletier *et al*, 1993). It seems unlikely that the described chondrocyte degeneration and death is due simply to one factor, and further investigation, similar to previous work (Pujol *et al.*, 1991; van Beuningen *et al.*, 1993) whereby combinations of growth factors and cytokines were added to cell cultures, could be one way forward. From the opposite direction, neutralising antibodies to various cytokines and growth factors are available and could be added to conditioned medium in an attempt to prevent the processes occurring. Filtration, column chromatography and even immunoblotting of the ECCM are further methods that could be used to separate

and possibly identify factors within the medium. Once identified, quantitation could simply be performed by using an enzyme linked immunosorbent assay (ELISA). An attempt to separate the factor(s) by HPLC was briefly attempted, though time limitations prevented further analysis.

Despite the alterations in morphology following ECCM treatment, there is expression of type II collagen throughout the culture period, which is associated with the chondrocytic phenotype (Solursh *et al.*, 1982). The loss of type X collagen indicates a loss of hypertrophy, as type X collagen is only produced by chondrocytes in a hypertrophic state (Kielty *et al.*, 1985; Schmid & Linsenmayer, 1987; Kirsch & von der Mark, 1991; Reichenberger *et al.*, 1991. Treatment with ECCM may terminate or reverse hypertrophy, though the effect is not immediate and is only very apparent after 7 days. This specific loss of type X collagen suggests a differential regulation of collagens, which has been demonstrated by O'Keefe *et al.*, (1997), whereby parathyroid hormone-related protein was shown to regulate type X collagen expression in chick chondrocytes without any significant effect on type II collagen expression.

The result for type I collagen is not clear, but there is no evidence for a particular phenotypic switch to type I collagen, as is seen when chondrocytes dedifferentiate into fibroblasts (Mayne *et al.*, 1976; Kirsch *et al.*, 1992; Bonaventure *et al.*, 1994). Therefore the initial change in morphology is not the same process as dedifferentiation.

As there seems to be little qualitative change in collagen expression, the next step would be to examine the proteoglycan/GAG expression of ECCM treated chondrocytes. Proteoglycan turnover in cartilage is known to be much more rapid than that of collagen (Mankin, 1974a) and indications from alcian blue staining is that ECCM treatment causes a substantial loss of proteoglycan production compared to equivalent controls. Histochemical (Christensen & Reimann, 1980) and biochemical (Mankin, 1974b; Mankin *et al.*, 1981; Ryu *et al.*, 1984; Hamerman, 1989) techniques have shown that a change in GAG is linked to cartilage degeneration. It would be of value to see if the effects of the vasculature are responsible for any changes in the production of cartilage proteoglycans or GAG's in cultured chondrocytes. Simple chemical staining of cultures *in-situ* with the alcian blue CEC technique (Scott & Dorling, 1965) combined with the ABBA technique (Zimmerman *et al.*, 1994) to differentiate specific GAG's, may provide indications of changes, particularly if there is a loss of cartilage specific GAG's.

Quantitatively, the effects are rather more dramatic. The rapid loss of incorporation of radiolabelled precursor into collagenous proteins shows a decrease in the amount of protein released into the medium from the cell. Due to the presence of mRNA transcripts in most samples, the production of collagen would appear to be inhibited at the translational level. However, subsequent work is needed to investigate whether this is due to an inability of the cells to release the collagens, maintaining them intracellularly. Recovery of intracellular

proteins could be performed, for instance, by scraping the cells off their base and solubilisation in SDS (Horton & Hassell, 1986) or by homogenisation of the cells (Schor *et al.*, 1982), before similar precipitation procedures as described earlier

At certain time points, 7 days in particular, the lack of mRNA could explain the lack of protein. However, caution must be applied in this interpretation. The RT-PCR reactions performed were not quantitative, meaning that a decrease in band intensity does not actually attest to a decrease in mRNA amounts, though it may be an indicator, particularly as loss of intensity of mRNA species consistently occurs at 7 days, and the intensity of the G-3-DPH band did not visually alter. It has been shown previously that collagen regulation often occurs at the gene level (Mallein Gerin *et al.*, 1990; Thomas *et al.*, 1990). Apart from samples at 7 days, there was little difference in mRNA production for the collagen types studied, indicating that any control on collagen production in ECCM treated cells was not at the transcriptional level. Taken purely as a qualitative result, the RT-PCR could even be intrepreted as showing no differences in mRNA production, except perhaps for type I collagen. Even any loss of transcriptional activity was not permanent at 7 days of culture. Increased band intensity at 10 days may show a reversal of the inhibitory effects, which could be an attempt at repair, and there is a slight increase in $^{3}$H-proline incorporation/cell in terms of collagenous proteins from 7 days to 10 days. Repair attempts are seen in human OA cartilage by the

formation of chondrones and overproduction of cartilage components (Mankin *et al.*, 1971; Mankin, 1974b; Howell *et al.*, 1976; 1981; Lipiello *et al.*, 1977; Ryu *et al.*, 1984; Grushko *et al.*, 1989; Lafeber *et al.*, 1992b, 1993; Archer, 1994). Overall, the late stage sequence of events may be some form of repair response, as a last attempt to survive. A repair response was also seen in periosteal-free stage 35/36 hypertrophic zones, after they were grafted onto the CAM. Identical non-CAM grafted zones showed very degenerate chondrocytes. After CAM grafting, the cells had returned to a more chondrocytic phenotype and generally appeared much healthier. This provides direct evidence that there is a potential for reversal of vascular derived degenerative effects in chondrocytes. Elucidation of collagen types by degenerate and repaired cells *in-situ* would need to be studied to determine any differences in collagen expression, via immunohistochemistry for protein expression, and by *in-situ* hybridisation to examine mRNA species.

Regardless of any ambiguity in the effect of the ECCM on the chondrocytes, they are all virtually dead by 10-12 days of culture. Hypertrophic chondrocytes are thought to be terminally differentiated cells (Pacifici *et al.*, 1990) which degenerate and die by necrosis (Fell, 1925; Roach *et al.*, 1995). Alternative views state that chondrocytes expire by apoptosis (Descalzi Cancedda *et al.*, 1992; Hatori *et al.*, 1995; Roach *et al.*, 1995). In developing cartilage, chondrocyte apoptosis has been detected in the hypertrophic zone of chick

embryos along the invasion rim (Gibson *et al.*, 1995a) and in the growth plate of long bones (Hatori *et al.*, 1995; Zenmyo *et al.*, 1996). Morphological indications of 7-10 day ECCM treated cells suggest apoptosis, in that they were shrunken and contained a crenated cell membrane (Cohen, 1996). However, there was no production of degraded DNA ladders which would be expected if apoptosis was occuring (Koopman *et al.*, 1994; Cohen, 1996). Control cells morphologically demonstrated no evidence of apoptosis, and showed no indications of DNA laddering. The results with annexin V binding did not elucidate much since both control and treated cells had high levels of binding, and in some cases, the level was greater than cells treated with staurosporin, an inducer of apoptosis (Leist *et al.*, 1997). Since annexin V is a homologue of anchorin CII (Kirsch & Pfaffle, 1992), a collagen binding protein, it is possible that the high annexin V binding could be due to the binding of collagen fragments still present on trypsinised cells. It may well be that a proportion of the cells in both control and ECCM cultures do undergo apoptosis, but to detect this, the annexin V method would need to be optimised for these cells. For instance, collagenase treatment of chondrocytes to ensure degradation of any surface bound collagen, before annexin V incubation may give a more accurate result, although even in freshly dissociated cells, 30% of the cells still bound annexin V. Overall, the technique is extremely limited in this particular application due to the fundamental binding properties of annexin V. Future investigation of this problem will probably require different techniques, such as

the TUNEL technique (Transferase-mediated, biotin dUTP Nick End Labelling) for end labelling of DNA breaks which occur during apoptosis, which has been used previously (Gibson *et al.*, 1995a, Bronckers *et al.*, 1996). This technique was attempted, but due to time limitations, the technique could not be optimised and no meaningful results were obtained. Further work with this technique would hopefully show whether ECCM causes apoptosis in hypertrophic chondrocytes, and if so, there would be a need for quantification to determine what percentage of cells are encouraged to undergo apoptosis by the ECCM. The flow cytometer has the advantage of being able to do this. New technology has been developed in the form of laser scanning cytometry. This technique allows laser scanning of cells on slides, for instance, in the form of cytospins, and presents the results in a similar format to that of a flow cytometer, thereby allowing quantification of cells that take up a particular fluorescent marker. This methodolgy provides opportunity for adaption for use with the TUNEL technique.

The last model of cartilage vascularisation developed here was that of human cartilage on the CAM. Samples of OA and comparatively healthy cartilage were grafted onto the CAM in an identical manner to the chick cartilage. Articular cartilage is thought to be anti-angiogenic (Moses *et al.*, 1990; Moses, 1993). However, when grafted onto the CAM, which is accepted to be a qualitative bioassay for angiogenesis (Nguyen *et al.*, 1994), both OA and more suprisingly, healthy cartilage became enveloped in CAM tissue containing

numerous blood vessels (Figure 6.1). The pattern of histochemical staining, of the vascular invasion, of human OA cartilage on the CAM was remarkably similar to that observed in developing chick cartilage. There was a loss of staining of safranin-O, toluidine blue and alcian blue CEC at all concentrations of $MgCl_2$. The same cells were also seen in the invasion, including the darkly staining granular cells. Mankin *et al.*, (1971) described a histological/ histochemical technique to grade the severity of the disease process in OA. An increase in grade correlates with an increase in severity and also an increase in the uptake of precursors of collagen, proteoglycan and DNA. However, at a point on this scale, grade 10, there is a marked fall off in incorporation of precursors, indicative of a failure of the cells to maintain a repair response. It would be of interest to see if there is a correlation between grade of severity and loss of avascularity. All of the OA cartilage that was CAM grafted, was from late stages of the disease i.e. from a stage where joint replacement was necessary. If cartilage could be obtained from a range of stages of the disease, from early to late, and grafted, sections could be examined for vascularisation, and also stained with haemotoxylin and eosin and safranin-O fast green to determine if there is a point on the scale where the cartilage loses its ability to maintain avascularity.

Taken in association with the observation that normal articular cartilage was surrounded by blood vessels, but was never vascularised, these data suggest that OA cartilage loses its anti-angiogenic capacity and that the data are real and

not due to the culture technique.

Immunostaining for types I, II, III and X collagen in healthy and OA human articular cartilage has been performed previously, showing various staining patterns (Adam & Deyl, 1983; Aigner *et al.*, 1992; von der Mark *et al.*, 1992; Aigner *et al.*, 1993a & b; Nerlich *et al.*, 1993; Hollander *et al.*, 1994). Similar patterns of staining are described here indicating that the invasion does not have a profound effect on overall collagen expression in human OA cartilage, there being no outstanding difference between grafted and non-grafted OA cartilage. However, a further reason for the immunostaining was to determine whether there was any particular change in collagen linked specifically to the invasive vessels. There was specific deposition of types I and X around the invasive vessel. The source of these collagens cannot be determined with certainty. The anti-type X antibody is a monoclonal human antibody, suggesting that the type X around the invasion is derived from the cartilage. The source of the type I collagen could be either chick or human as the antibody cross-reacts with both.

The reason for the deposition of these collagens certainly needs further investigation. Type I collagen is associated with endothelial cells and angiogenesis (Rooney *et al.*, 1993) and type X collagen is hypothesised to have many roles, including a role in the calcification/mineralistion of cartilage (Schmid & Linsenmayer, 1987; Schmid *et al.*, 1990; Iyama *et al.*, 1991; Rooney & Kumar, 1993; Wallis, 1993). Mutations in the type X collagen gene caused

mice to develop skeletal deformities (Jacenko *et al.*, 1993), indicating that type X collagen is a necessity for normal skeletal development. Despite this, type X collagen null mice generated by Rosati *et al.*, (1994) were shown to develop normally with no gross abnormalities. However, in the absence of mRNA transcripts for type X collagen in these null mice, staining with anti-type X antibodies still showed the presence of type X collagen throughout the growth plate (Rosati *et al.*, 1994). It was suggested that the antibody was cross reacting with another component, though an identity was not suggested. It is very possible that this unknown component is type VIII collagen, due to the similarity in structure (Ayad *et al.*, 1994) and the fact that the anti-type X antibody used here is known to cross react with type VIII collagen (P. Rooney, personal communication). It is may be the case that type VIII collagen is able to replace type X collagen in its complete absence, though if a mutated form of type X collagen is expressed, type VIII is not used.

It is hoped that this model may serve as a valuable tool to study changes within articular cartilage undergoing vascularisation. *In-situ*, it takes many years for OA to develop and the processes involved in the subsequent degeneration to occur (Mankin, 1974b; Lohmander, 1994). The model used here shows a very rapid invasion of the cartilage. Within 5 days, there are often extensive blood vessels within the cartilage matrix, invasion is therefore comparatively rapid and can be examined within days of obtaining the sample.

How closely this invasion mimics that occurring during the disease

process remains to be fully determined. During OA, the role of vascular invasion is to erode the cartilage and allow endochondral ossification to occur, this occurs very slowly and the cartilage is eroded by calcification and new bone formation in the radial zone (Howell *et al.*, 1976). Although cartilage placed on the CAM never showed any histological evidence of mineralisation or endochondral ossification, as it does *in-situ*, the model may still be of value in terms of studying changes occuring within the matrix during the invasion of blood vessels.

There is one particular difficulty in working with human cartilage, the availability of truly healthy cartilage for use as control tissue. Cartilage from non-diseased sources, such as the ankle joint and hips from fractured neck of femurs, though not osteoarthritic, are still likely to show symptoms of cartilage damage as articular cartilage degeneration can begin at a relatively early age (Howell *et al.*, 1976). A source of truly healthy human articular cartilage is still required to allow certainty in any results obtained when investigating changes due to OA, or any other cartilage disease.

## FINAL COMMENTS

There is little doubt, from the work presented here, that the vasculature plays a major, if not central role in the degeneration and removal of cartilage, both as a developmental process in bone formation, and in the pathological process of OA. Soluble factors arising from the endothelium cause degeneration of

chondrocytes and a subsequent decrease of matrix components. The main aim now is to identify the factors involved in causing this degeneration, and to characterise its effects on not just the collagens, as has been attempted here, but to extend the scope to the other components of the cartilage matrix. Any effect that may occur on the expression of MMP's and TIMP's, responsible for the maintenance and destruction of cartilage, will also need subsequent evaluation. This information may shed light on the reasons for cartilage breakdown during OA, and help us to understand at least some of the reasons why cartilage becomes vascularised during OA.

## FUTURE DIRECTIONS

Definition of the role played by the periosteum, and any interplay it has with the developing hypertrophic cartilage may shed further light on the factors involved in the initiation of hypertrophic cartilage vascularisation. Of particular use would be the ability to define sub-populations of hypertrophic chondrocytes at the different stages of invasion, i.e. whether some do have an intrinsic ability to become vascularised and others do not.

Further investigation of the effects of the soluble factors released by vascular cells may help determine the true fate of hypertrophic chondrocytes and could aid in our understanding of the effects of the vasculature on both developing and pathological cartilage. If sub-populations of chondrocytes can be defined in terms of their response and fate when exposed to the vasculature,

it may help us understand better the reasons for cartilage vascularisation and the role it has to play in disease processes.

Finally, further examination of the deposition of collagens during the vascularisation of human cartilage would aid our understanding of the role of these collagens and the reason for their location. In particular, determination of whether type VIII is present and how much it is involved with type X collagen, and what is their specific role in the vascularisation process.

# APPENDIX I

# General Materials & Methods

*Eggs*

Fertilised White Leghorn chicken eggs were obtained from a registered breeder. All eggs were wiped with 70% methanol and incubated in a humidified, tilting egg incubator at 37°C. At 3 days the eggs were windowed by cutting a small hole into the side of the egg, which was subsequently sealed with sellotape. The embryos were staged according to Hamburger and Hamilton (1951) and the eggs returned to the incubator until the desired stages of development were reached.

*Harvesting of Cartilage*

Harvesting of cartilage was performed under aseptic conditions. When the desired stage was reached, embryos were removed from eggs and placed into sterile petri dishes in unsupplemented α-MEM medium (Gibco Life Technologies, Paisley, U. K.). Cartilage long bone rudiments were removed from both wings and legs and cleared of all connective tissue. Intact long bone rudiments and individual hypertrophic and rounded cell zones, were excised using a scalpel under a dissecting microscope.

*CAM Grafting*

Host eggs were used at 10 days of age and were previously windowed at 3 days of age as described above. Tissue to be CAM grafted was carefully laid onto the CAM with a pair of forceps or a spatula and the window resealed with sellotape under aseptic conditions. The eggs were returned to the incubator for

the desired incubation time for the graft.

*Preparation of Hypertrophic Chondrocytes*

Pure hypertrophic cartilage was obtained by removal of the periosteum. This was achieved in one of two ways. All hypertrophic cell zones from stage 34 rudiments and those from the ulna, radius and humerus of stage 36 zones were squeezed in the centre with forceps, expelling cartilage from the ends. Periostea from the larger zones of stage 36 zones i.e. the tibia and femur were carefully cut along their length with a scalpel or with sharp forceps, and gently teased apart to expose the cartilage. The cartilage was digested overnight in collagenase (type 1A, Sigma) at $^1/_2$-1mg/ml in α-MEM supplemented with 10% foetal bovine serum (FBS), L-glutamine (1.4 mM), Penicillin (50 IU/ml) and Streptomycin (50 μg/ml) (all Gibco), to release the cells, which were collected by centrifugation at 2,000 rpm for 5 minutes, rinsed and re-centrifuged before counting and plating as required in the same medium.

*Endothelial Cell Preparation*

Bovine aortic endothelial cells (BAEC) were extracted by an adapted method from Kumar *et al.,* (1992). Fresh bovine aortas were obtained from a local abattoir. The first 60-70cm of the aorta was removed and the excess fat and tissue were removed. Both ends, plus the intercostal vessels were tied and collagenase solution (0.5mg/ml) was injected into the aorta and incubated at 37°C for 20 minutes. The collagenase solution was then withdrawn and

centrifuged at 2,000 rpm for 5 minutes, the cells washed twice with fresh medium (α-MEM (Gibco) supplemented with 10% FBS (Gibco) penicillin/streptomycin (Gibco) and L-glutamine (Gibco) as for the chondrocytes) and plated into 80cm$^2$ flasks (Costar) in the same medium. Cells were fed every second day.

Contaminating fibroblastic cells extracted along with the endothelial cells were removed by serial dilution to theoretical maximum of 10 cells/ml, and 0.1 ml aliquots were plated into 96 well multiwell plates (Nunc). Cells were incubated until confluency was acheived and those wells containing cells showing only the classic cobblestone morphology of endothelial cells were passaged and pooled. These cells were then cultured in the same medium as described and passaged to their fifth passage before use, and used between passages 5 and 10 (Iruela-Arispe *et al.*, 1991). Batches of low passage number cells were frozen in FBS containing 10% di-methyl sulphoxide in liquid nitrogen and stored until needed. This ensured all BAEC were from the same animal.

To check that the cells were indeed endothelial in origin, they were immunostained with a rabbit anti-human von Willebrand Factor antibody (Dako), which did cross react slightly with the bovine cells as seen by visualisation with a biotinylated swine anti-rabbit secondary antibody (Dako), ABComplex (Dako) and DAB (Sigma).

*Conditioned Medium*

Endothelial cells from 5th passage or greater (up to 10 passages) were allowed to grow to confluency (approx. 4-5 days). Medium from confluent and sub-confluent cells was removed every third day and passed through a 2μm filter (Acrodisc) before pooling for use immediately, or storage at -20°C.

*Paraffin Wax Histology*

Tissue for paraffin wax embedding and histology was fixed for up to a week in 10% buffered formalin before processing up through graded alcohols (70%, 90%, 100%) into xylene and finally into paraffin wax under vacuum. Tissue sections were cut at 5-7μm on a base sledge microtome (Anglia Scientific) before mounting on silane (Sigma) coated glass slides. For histological/histochemical staining, sections were dewaxed in Histoclear (National Diagnostics) before hydrating through a series of graded alcohols into water prior to staining.

*Histological/Histochemical staining*

*Haemotoxylin and Eosin (H&E)*- Slides were hydrated through a series of alcohols stained in Mayers Haemotoxylin (Appendix II) for 10 minutes before rinsing in water and differentiating briefly in 1% acid alcohol (Appendix II) for 1-2 seconds and rinsed again in water to 'blue'. The sections were then stained in 0.5% eosin (yellowish) (Appendix II) for 20 seconds before a final brief rinse in water and rapidly dehydrated up to histoclear via a series of graded alcohols.

Sections were mounted in DPX (Fisons)

*Safranin-O for staining of proteoglycans*- Sections were placed in safranin-O (Appendix II) for 10 minutes before rinsing in water, dehydrating and mounting as for H&E.

*Toluidine Blue for metachromasia of ECM*- Slides were placed in aqueous 1% toluidine blue for 1 minute then rinsed in water before dehydrating and mounting as above.

*Alcian Blue CEC for staining glycosaminoglycans (GAG) (Scott & Dorling, 1965)*- Sections were stained overnight in 0.05% alcian blue (Appendix II) containing varying concentrations of $MgCl_2$ (0.06M for all acid mucins, 0.7M for heparan and keratan sulphate, 0.9M for keratan sulphate only). The slides were rinsed, dehydrated and mounted as before.

# APPENDIX II

# Working Solutions

*10% Neutral Buffered Formalin:*

100mls formalin (40% formaldehyde)

6.5g dibasic anhydrous sodium phosphate (BDH)

4g monobasic sodium phosphate monohydrate (BDH)

900mls distilled water

*Acetate Buffer (for hyaluronidase)*

50mM NaAc and 2mM NaCl in distilled water.

## *Histology & Histochemistry*

*Alcian Blue:*

0.05% Alcian blue (BDH) in 3% Acetic acid (Fisons).

*Alcian Blue CEC (Scott & Dorling, 1965):*

0.05% alcian blue (BDH) in 0.2M acetate buffer (200mM NaAc + 100mM NaCl per 100ml water, pH to 5.8). $MgCl_2$ added to required molarity (0.06M, 0.7M or 0.9M).

*Haemotoxylin:*

2g of haemotoxylin (BDH) + 10 mls of 99% alcohol

2,000mls of distilled water is warmed, to which is added 100g aluminium potassium phosphate (Sigma), 0.4g sodium iodate (Sigma) and 100g chloral hydrate (Sigma) until dissolved. The haemotoxylin/alcohol is then added. Solution is ready for immediate use.

***Safranin-O:***

0.5% safranin-O (Sigma)in 0.1M Acetate buffer pH 4.6 (100mM sodium acetate (Sigma) and 50mM sodium chloride (Sigma) in distilled water, pH to 4.6).

## *Protein Electrophoresis*

### *8% Polyacrylamide Gel (B. D. Hanes, 1988):*

8ml of acrylamide/bis-acrylamide stock (30% acrylamide (Sigma) and 0.8% bis-acrylamide (BDH) in distilled water)

3.75ml resolving gel buffer stock (3M Tris HCl pH8.8 - 36.3g Tris (Sigma) 48ml 1M HCl bring to 100ml final volume with distilled water, pH to 8.8)

10% SDS (10% SDS (BDH) in distilled water)

1.5ml of 1.5% ammonium

15µl TEMED (Sigma)

16.45 mls distilled water

### *10x Polyacrylamide Gel Running Buffer (Reservoir Buffer):*

30.3g Tris (Sigma) and 144.2g glycine (BDH) in 1,000ml distilled water. For working solution, dissolve 10x in deionised water.

***Gel Fixing Solution:***

10% acetic acid

25% isopropanol

65% deionised water.

***Polyacrylamide Gel Loading Buffer (3x Stock) (Adapted from Laemmli, (1970)):***

10mg bromophenol blue (BDH)

2g SDS (BDH)

6.25ml 1M Tris (pH 6.8)

2.05ml deionised water

Add solution to 10ml of glycerol. For working solution, dilute 3 times with deionised water.

***Western Blot Transfer Buffer (Towbin et al., (1979)):***

2.93g glycine (BDH)

5.81g Tris (Sigma)

0.375 g SDS (BDH)

800ml distilled water

Immediately before use, add 20% v/v methanol.

## *RT-PCR*

### *50x TAE:*

121g Tris (Sigma)

57.1ml analytical grade glacial acetic acid (BDH)

50ml 0.5 M EDTA (pH 8.0)

Make to 300ml with deionised water and pH to 7.65 with concentrated hydrochloric acid. Finally make up to 500ml with deionised water. For working solution, dilute 50 times with deionised water.

### *Agarose Gel Loading Buffer (5x Stock):*

10mg of bromophenol blue (BDH)

10 mg of xylene cyanol (BDH)

8ml deionised water

10ml glycerol

2ml of 50x TAE

### *PCR Buffer:*

PCR buffer was prepared as below giving a 5/3 final concentration for use in PCR:

| | Stock Concentration | Volume of Stock | Final Concentration |
|---|---|---|---|
| Tris .HCl pH 8.8* | 1M | 375µl | 45mM |
| Ammonium sulphate (Sigma) | 1M | 93µl | 11mM |
| Magnesium Chloride | 1M | 37.5µl | 4.5mM |

| (Sigma) | | | |
|---|---|---|---|
| dNTP's (Pharmacia) | 100mM | 20µl of each | 200µM |
| BSA (Boerhinger Mannheim) | 20mg/ml | 45.8µl | 113µg/ml |
| β-mercaptoethanol (Sigma) | 14.4M | 4µl | 11.16mM |
| EDTA pH 8.0** | 0.5M | 0.1µl | 4.4µM |
| Ultra Pure Water (Sigma) | -- | 4364.6µl | -- |

Reagents were mixed and stored as 500µl aliquots at -20°C.

* 1M Tris (ICN) in distilled water, pH to 8.8 with concentrated HCl .

** 0.5M EDTA (Sigma) in distilled water, pH to 8.0.

***RT-PCR dNTP Mix:***

10µl each of dCTP, dGTP, dTTP and dATP (Promega) added to 60µl of ultrapure water (Sigma). Aliqouts of 10µl were stored at -20°C until needed.

***RNase Solution:***

50mg/ml RNase (Sigma) in 10mM Tris (pH 7.5) containing 15mM sodium chloride (Sigma).

# Bibliography

Adam M. & Deyl Z. (1983) Altered Expression of Collagen Phenotype in Osteoarthritis. *Clinica Chimica Acta* **133**, 25-32.

Ahrens P. B., Solursh M. & Reiter R. S. (1977) Stage Related Capacity for Limb Chondrogenesis in Cell Culture. *Dev. Biol.* **60**, 69-82.

Aigner T., Bertling W., Stoβ H., Weseloh G. & von der Mark K. (1993a) Independent Expression of Fibril-Forming Collagens I, II and III in Chondrocytes of Human Osteoarthritic Cartilage. *J. Clin. Invest.* **91**, 829-837.

Aigner T., Riechenberger E., Bertling W., Kirsch T., Stoβ H. & von der Mark K. (1993b) Type X Collagen Expression in Osteoarthritic and Rheumatoid Articular Cartilage. *Virchows Archiv B Cell Pathol.* **63**, 205-211.

Aigner T., Stoβ H., Weseloh G., Zeiller G. & von der Mark K. (1992) Activation of Type II Collagen Expression in Osteoarthritic and Rheumatoid Cartilage. *Virchows Archiv B Cell Pathol.* **62**, 337-345.

Alini M., Marriot A., Chen T., Abe S. & Poole A. R. (1996) A Novel Angiogenic Molecule Produced at the Time of Chondrocyte Hypertrophy During Endochondral Bone Formation. *Dev. Biol.* **176**, 124-132.

Anderson H. C. (1995) Molecular Biology of Matrix Vesicles. *Clin. Orthop. Rel. Res.* **314**, 266-280.

Archer C. W. (1994) Skeletal Development and Osteoarthritis. *Ann. Rheum. Dis.* **53**, 624-630.

Archer C. W., Cottrill C. P. & Rooney P. (1984) Cellular Aspects of Cartilage Differentiation and Morphogenesis. In: *Matrices and Cell Differentiation.* Eds. Kemp R. B. & Hinchcliffe J. R. New York: Alan R. Liss. pp 409-426.

Archer C. W., McDowell J., Bayliss M. T., Stephens M. D. & Bentley G. (1990) Phenotypic Modulation in Sub-Populations of Human Articular Chondrocytes *In-Vitro. J. Cell Sci.* **97**, 361-371.

Archer C. W., Rooney P. & Wolpert L. (1982) Cell Shape and Cartilage Differentiation of Early Chick Limb Bud Cells in Culture. *Cell Differentiation* **11**, 245-251.

Archer C. W., Rooney P. & Wolpert L. (1983) The Early Growth and Morphogenesis of Limb Cartilage. In: *Limb Development and Regeneration: Progress in Clinical and Biological Research 110A.* Eds Fallon J. F. & Caplan A. I. New York: Alan R. Liss. pp267-278.

Aspden R. M. & Hukins D. W. (1979) The Lamina Splendens of Articular Cartilage is an Artefact of Phase Contrast Microscopy. *Proc. R. Soc. Lond. Biol.* **206**, 109-113.

Aspden R. M. (1994) Fibre Reinforcing by Collagen in Cartilage and Soft Connective Tissues. *Proc. Royal Soc. Lond. B* **258 (1352)**, 195-200

Aspden R. M. (1996) Could Type X Collagen Have a Mechanical Function?. *Int. J. Exp. Pathol.* 77, A3-A4.

Aulthouse A. L. (1994) Prolonged Exposure of Human Chondrocytes to Ascorbic Acid Modifies Cellular Behaviour in Agarose Gel. *Anat. Rec.* **238**, 31-37.

Ayad S., Boot-Handford R. P., Humphries M. J., Kadler K. E. & Shuttleworth C. A. (1994) In: *The Extracellular Matrix: Facts Book.* Academic Press.

Bassleer C. T., Henrotin Y. E., Reginster J.-Y. L. & Franchimont P. P. (1992) Effects of Tiaprofenic Acid and Acetyl Salicylic Acid on Human Articular Chondrocytes in 3-Dimensional Culture. *J. Rheumatol.* **19**, 1433-1438.

Benninghoff A. (1925) Form und Bau der Gelenkknorpel in ihren Beziehungen zur Funktion. *Z. Zelleforsch U. Mikr. Anat.(Berlin).* **2**, 783-862.

Benya P. D. & Shaffer J. D. (1982) Dedifferentiated Chondrocytes Re-express the Differentiated Collagen Phenotype When Cultured in Agarose Gels. *Cell* **30**, 215-224.

Berry L., Grant M. E., McClure J. & Rooney P. (1992) Bone-Marrow-Derived Chondrogenesis *In-Vitro. J. Cell Sci.* **101**, 333-342.

Blanco F. J., Ochs R. L., Schwarz H. & Lotz M. (1995) Chondrocyte Apoptosis Induced by Nitric Oxide. *Am. J. Pathol.* **146**, 75-85.

Bonaventure J., Kadhom N., Cohen-Solal L., Ng K. H., Bourguignon J., Lasselin C. & Freisinger P. (1994) Reexpression of Cartilage-Specific Genes by Dedifferentiated Human Articular Chondrocytes Cultured in Alginate Beads. *Exp. Cell Res.* **212**, 97-104.

Bonen D. K. & Schmid T. M. (1991) Elevated Extracellular Calcium Concentrations Induce Type X Collagen Synthesis in Chondrocyte Cultures. *J. Cell Biol.* **115**, 1171-1178.

Bradbury P. & Gordon K. C. (1990) Connective Tissues and Stains. In *Theory*

*and Practice of Histological Techniques*. Eds. Bancroft J. D. & Stevens A. Churchill Livingstone. pp.119-142.

Brierley V. H., Ayad S. & Grant M. E. (1991) Types II, VI and IX collagens in Normal and Osteoarthritic Human Articular Cartilage. *Biochem. Soc. Trans.* **19**, 379S.

Bronckers A. L., Goei W., Luo G., Karsenty G., D'Souza R. N., Lyaruu D. M. & Burger E. H. (1996) DNA Fragmentation During Bone Formation in Neonatal Rodents Assessed by Transferase-Mediated End Labelling. *J. Bone Mineral Res.* **11**, 1281-1291.

Brown J. C. & Timpl R. (1995) The Collagen Superfamily. *Int. Arch. Allergy Immunol.* **107**, 484-490.

Brown R. A. & Weiss J. B. (1988) Neovascularisation and its Role in the Osteoarthritic Process. *Annals Rheum. Dis.* **47**, 881-895.

Bullough P. G. (1997) *Bullough & Vigorita's Orthopaedic Pathology* 3rd Edition. Mosby-Wolfe.

Burgeson R. E. & Hollister D. W. (1979) Collagen Heterogeneity in Human Cartilage: Identification of Several New Chains. *Biochem. Biophys. Res. Commun.* **87**, 1124-1131.

Burgeson R. E. & Nimni M. E. (1992) Collagen Types. Molecular Structure and Tissue Distribution. *Clin. Orthop. Rel. Res.* **282**, 250-272.

Caplan A. I. & Pechak D. G. (1987) The Cellular and Molecular Embryology of Bone Formation. In: *Bone and Mineral Research*, 5th edition. Ed W. A. Peck. Elsevier Science Publishers. pp 117-181.

Caplan A. I. (1990) Cartilage Begets Bone Versus Endochondral Myelopoiesis. *Clin. Orthop. & Rel. Res.* **261**, 257-267.

Carney S. L. (1991) Cartilage Research, Biochemical, Histologic and Immunochemical Markers in Cartilage, and Animal Models of Osteoarthritis. *Curr. Op. Rheumatol.* **3**, 669-675.

Carrino D. A. & Caplan A. I. (1985) Isolation and Characterisation of Proteoglycans Synthesised *In-Ovo* by Embryonic Chick Cartilage and New Bone. *J. Biol. Chem.* **260**, 122-127.

Castagnola P., Dozin B., Moro G. & Cancedda R. (1988) Changes in the Expression of Collagen Genes Show Two Stages in Chondrocyte Differentiation *In Vitro*. *J. Cell Biol.* **106**, 461-467.

Castagnola P., Moro G., Descalzi-Cancedda F. & Cancedda R. (1986) Type X Collagen Synthesis During *In-Vitro* Development of Chick Embryo Tibiae Chondrocytes. *J. Cell Biol.* **102**, 2310-2317.

Cawston T., Plumpton T., Curry V., Ellis A. & Powell L. (1994) Role of TIMP and MMP Inhibition in Preventing Connective Tissue Breakdown. *Annals New York Acad. Sci.* **732**, 75-83.

Chandrasekhar S., Harvey. A. K. & Stack S. T. (1993) Degradative and Repair Responses of Cartilage to Cytokines and Growth Factors Occur Via Distinct Pathways. In: *Joint Destruction in Arthritis and Osteoarthritis.* Eds. van den Berg W. B. *et al.* Birkhauser Verlag Basel. pp121-125.

Christensen S. B. & Reimann I. (1980) Differential Histochemical Staining of Glycosaminoglycans in the Matrix of Osteoarthritic Cartilage. *Acta Path. Microbiol. Scand. Sect A.* **88**, 61-68.

Clark J. M. (1985) The Organisation of Collagen in Cryofractured Rabbit Articular Cartilage. *J. Orthop. Res.* **3**, 17-29.

Closs E. I., Murray A. B., Schmidt J., Schon A., Erfle V & Gunter Strauss P. (1990) C-fos Expression Precedes Osteogenic Differentiation of Cartilage Cells *In Vitro. J. Cell Biol.* **111**, 1313-1323.

Cohen J. J. (1996) Apoptosis and its Regulation. In: *Mechanisms of Lymphocyte Activation and Immune Regulation VI.* Eds. Gupta & Cohen. Plenum Press, New York. pp11-20.

Cole A. A., Luchene L. J., Linsenmayer T. F. & Schmid T. M. (1992) The Influence of Bone and Marrow on Cartilage Hypertrophy and Degradation During 30-Day Serum-Free Culture of the Embryonic Chick Tibia. *Dev. Dynam.* **193**, 277-285.

Collins D. H. & McElligott T. F. (1960) Sulphate ($^{35}SO_4$) Uptake by Chondrocytes in Relation to Histological Changes in Osteoarthritic Human Articular Cartilage. *Ann. Rheum. Dis.* **19**, 318-330.

Curtis A. J., Ng C. K., Handley C. J. & Robinson H. C. (1992) Effect of Insulin-like Growth Factor on the Synthesis and Distribution of Link Protein and Hyaluronan in Explant Cultures of Articular Cartilage. *Biochim. Biophys. Acta* **1135**, 309-317.

Dean D. D. (1991) Proteinase Mediated Cartilage Degradation in Osteoarthritis. *Sem. Arth. Rheum.* **20** (suppl 2), 2-11.

Dean D. D., Martell-Pelletier J. & Pelletier J. P. (1989) Evidence for Metalloproteinase and Metalloproteinase. Inhibitor Imbalance in Human Osteoarthritic Cartilage. *J. Clin. Invest.* **84**, 678-685.

Descalzi Cancedda F., Gentili C., Manduca P. & Cancedda R. (1992) Hypertrophic Chondrocytes Undergo Further Differentiation in Culture. *J. Cell Biol.* **117**, 427-435.

DeSimone D. P. & Reddi A.H. (1992) Vascularisation and Endochondral Bone Development: Changes in Plasminogen Activator Activity. *J. Orthop. Res.* **10**, 320-324.

Dhawan R. R. & Beebe D. C. (1994) Differential Localization of Collagen Type IX Isoform Messenger RNAs During Early Ocular Development. *Investigative Opthalmology & Visual Science* **35**, 470-478.

Dietz U., Aigner T., Bertling W. M. & von der Mark K. (1993) Alterations of Collagen mRNA Expression During Retinoic Acid Induced Chondrocyte Modulation: Absence of Untranslated Alpha 1(I) mRNA in Hyaline Chondrocytes. *J. Cell. Biochem.* **52**, 57-68.

Dijkgraaf L. C., de Bont L. G. M., Boering G. & Liem R. S. B. (1995) Normal Cartilage Structure, Biochemistry and Metabolism. *J. Oral. Maxillofac. Surg.* **53**, 924-929.

Dunham J., Shackleton D. R. & Billingham M. E. (1988) A Reappraisal of the Structure of Normal Canine Articular Cartilage. *J. Anat.* **157**, 89-99.

Ekanayake S. & Hall B. K. (1994) Hypertrophy is not a Prerequisite for Type X Collagen Expression or Mineralisation of Chondrocytes derived from Cultured Chick Mandibular Ectomesenchyme. *Int. J. Dev. Biol.* **38**, 683-694.

Eyre D. R. & Muir H. (1976) Types 1 and 2 Collagens in Invertebral Disc. Interchanging Radial Distributions in Annulus Fibrosus. *Biochem. J.* **157**, 267-270.

Eyre D. R. (1991) The Collagens of Articular Cartilage. *Sem.Arth. Rheum.***21**,(suppl. 2), 2-11.

Eyre D. R., Wu J-J. & Apone S. (1987) A Growing Family of Collagens in Articular Cartilage: Identification of 5 Genetically Distinct Types. *J. Rheumatol.* **14**, 25-27.

Farquharson C. & Whitehead C. C. (1994) Differentiation and Mineralisation in Chick Chondrocytes Maintained in a High Cell Density Culture: A Model for Endochondral Ossification. *In Vitro Cell. Dev. Biol.* **31**, 288-294.

Fell H. B. (1925) The Histogenesis of Cartilage and Bone in the Long Bone Rudiments of the Embryonic Fowl. *J. Morphology and Physiol.* **40**, 417-459.

Fisher C., Gilbertson-Beadling S., Powers E. A., Petzold G., Poorman R. & Mitchell M. A. (1994) Interstitial Collagenase is Required for Angiogenesis *In Vitro. Dev. Biol.* **162**, 499-510.

Fitch J. M., Gordon M. K., Gibney E. P. & Linsenmayer T. F. (1995) Analysis of Transcriptional Isoforms of Collagen Types IX, II and I in the Developing Avian Cornea by Competitive Polymerase Chain Reaction. *Dev. Dynam.* **202**, 42-53.

Fitch J. M., Mentzer A., Mayne R. & Linsenmayer T. F. (1989) Independent Deposition of Collagen Types II and IX at Epithelial-Mesenchymal Interfaces. *Development* **105**, 85-95.

Folkman J. (1995) Angiogenesis in Cancer, Vascular, Rheumatoid and other Diseases. *Nature Medicine* **1**, 27-31.

Fosang A. J., Last K. & Maciewicz R. A. (1996) Aggrecan is Degraded by Matrix Metalloproteinases in Human Arthritis. Evidence That Matrix Metalloproteinase and Aggrecanase Activities can be Independent. *J. Clin. Invest.* **98**, 2292-2299.

Freije J. M., Dietz-Itza I., Balbin M., Sanchez L. M., Blasco R., Tolivia J. & Lopez-Otin C. (1994) Molecular Cloning and Expression of Collagenase-3, a Novel Human Matrix Metalloproteinase Produced by Breast Carcinomas. *J. Biol. Chem.* **269**, 16766-16773.

Galotto M., Campanile G., Robino G., Descalzi-Cancedda F., Bianco P & Cancedda R. (1994) Hypertrophic Chondrocytes Undergo Further Differentiation to Osteoblast-Like Cells and Participate in the Initial Bone Formation in Developing Chick Embryo. *J. Bone Mineral Res.* **9**, 1239-1249.

Gardner D. L. (1994) Problems and Paradigms in Joint Pathology. *J. Anat.* **184**, 465-476.

Gauss V. & Muller P. K. (1981) Change in the Expression of Collagen Genes in Dividing and Non-dividing Chondrocytes. *Biochim. Biophys. Acta* **652**, 39-47.

Genge B. R., Wu L. N. & Wuthier R. E. (1990) Differential Fractionation of Matrix Vesicle Proteins. Further Characterisation of the Acidic Phospholipid-Dependent $Ca^{2+}$ Binding Proteins. *J. Biol. Chem.* **265**, 4703-4710.

Gerstenfeld L. C., Finer M. H. & Boedtker H. (1989) Quantitative Analysis of Collagen Expression in Embryonic Chick Chondrocytes Having Different Developmental Fates. *J. Biol. Chem.* **264**, 5112-5120.

Gibson G. J., Kohler W. J. & Schaffler M. B. (1995a) Chondrocyte Apoptosis in Endochondral Ossification of Chick Sterna. *Dev. Dynam.* **203**, 468-476.

Gibson G. J., Lin D. L., Schaffler M. B. & Kimura J. H. (1995b) Endochondral Resorption of Chick Sterna in Culture. *J. Orth. Res.* **13**, 542-552.

Gibson G. J., Schor S. L. & Grant M. E. (1982) Effects of Matrix Macromolecules on Chondrocyte Gene Expression: Synthesis of a Low Molecular Weight Collagen Species by Cells Cultured Within Collagen Gels. *J. Cell Biol.* **93**, 767-774.

Goldring M. B., Birkhead J., Sandell L. J., Kimura T. & Krane S. M. (1988) Interleukin-1 Suppresses Expression of Cartilage-specific Types II and IX Collagens and Increases Types I and III Collagens in Human Chondrocytes. *J. Clin. Invest.* **82**, 2026-2037.

Goldring M. B., Fukuo K., Birkhead J. R., Dudek E. & Sandell L. F. (1994) Transcriptional Suppresion by Interleukin-1 and Interferon-Gamma of Type II Collagen Gene Expression in Human Chondrocytes. *J. Cell Biochem.* **54**, 85-99.

Gordon M. K., Foley J. W., Birk D. E., Fitch J. M. & Linsenmayer T. F. (1994) Type V Collagen and Bowmans Membrane. *J. Biol. Chem.* **40**, 24959-24966.

Gould R. P., Selwood L., Day A. & Wolpert L. (1974) The Mechanism of Cellular Orientation During Early Cartilage Formation in the Chick Limb and Regenerating Amphibian Limb. *Exp. Cell Res.* **83**, 287-296.

Granda J. L., Lande M. A. & Karvonen R. L. (1990) A Human Cartilage Metalloproteinase with Elastolytic Activity. *Conn. Tiss. Res.* **24**, 249-263.

Grandolfo M., D'Andrea P., Paoletti M., Martina M., Silvestrini G., Bonucci E. & Vittur F. (1993) Culture and Differentiation of Chondrocytes Entrapped in Alginate Gels. *Calcif. Tissue Int.* **52**, 42-48.

Grushko G., Schneiderman R. & Maroudas A. (1989) Some Biochemical and Biophysical Parameters for the Study of the Pathogenesis of Osteoarthritis: A Comparison Between the Processes of Ageing and Degeneration in Human Hip Cartilage. *Conn. Tiss. Res.* **19**, 149-176.

Grynpas M. D., Alpert B., Katz I., Lieberman I. & Pritzker K. P. H. (1991) Subchondral Bone in Osteoarthritis. *Calcif. Tissue Int.* **49**, 20-26.

Guerne P. A., Sublet A. & Lotz M. (1994) Growth Factor Responsiveness of Human Articular Chondrocytes: Distinct Profiles in Primary Chondrocytes, Subcultured Chondrocytes and Fibroblasts. *J. Cell. Physiol.* **158**, 476-484.

Gunther M., Haubeck H.-D. Van de Leur E., Blaser J., Bender S., Gutgemann I.,Fischer D.-C., Tschesche H., Greiling H., Henrich P. C. & Graeve L. (1994) Transforming Growth Factor Beta-1 Regulates Tissue Inhibitor of Metalloproteinase-1 Expression in Differentiated Human Articular Chondrocytes. *Arth. Rheum.* **37**, 395-405.

Hallmann R., Feinberg R. N., Latker C. H., Sasse J. & Risau W. (1987) Regression of Blood Vessels Precedes Cartilage Differentiation During Chick Limb Development. *Differentiation* **34**, 98-105.

Hamburger V. & Hamilton H. L. (1951) A Series of Normal Stages in the Development of the Chick Embryo. *J. Morph.* **88**, 49-92.

Hamerman D. (1989) The Biology of Osteoarthritis. *New Eng. J. Med.* **320**, 1322-1330.

Hanes B. D. (1988) An Introduction to Polyacrylamide Gel Electrophoresis. In *Gel Electrophoresis of Proteins: A Practical Approach.* Eds. Hanes B. D. & Rickwood D. IRL Press, Oxford pp.1-91.

Hatori M., Klatte K. J., Teixeira C. C. & Shapiro I. M. (1995) End Labelling Studies of Fragmented DNA in the Avian Growth Plate: Evidence of Apoptosis in Terminally Differentiated Chondrocytes. *J. Bone Mineral Res.* **10**, 1960-1968.

Hauselmann H. J., Fernandes R. J., Mok S. S., Schmid T. M., Block J. A., Aydelotte M. B., Kuettner K. E. & Thonar E. J.-M. A. (1994) Phenotypic Stability of Bovine Articular Chondrocytes After Long-Term Culture in Alginate Beads. *J. Cell Sci.* **107**, 17-27.

Heinegard D. & Oldberg A. (1989) Structure and Biology of Cartilage and Bone Non-collagenous Macromolecules. *FASEB J.* **3**, 2042-2051.

Heinegard D. & Saxne T. (1991) Molecular Markers of Processes in Cartilage in Joint Disease. *Br. J. Rheumatol.* **30** (Suppl 1), 21-24.

Heron G. S., Banda M. J., Clark E. J., Gavidovic J. & Werb Z. (1986a) Secretion of Metalloproteinases by Stimulated Capillary Endothelial Cells II. Expression of Collagenase and Stromelysin Activities is Regulated by Endogenous Inhibitors. *J. Biol. Chem.* **261**, 2814-2818.

Heron G. S., Werb Z., Dwyer K. & Banda M. J. (1986b) Secretion of Metalloproteinases by Stimulated Capillary Endothelial Cells I. Production of Pro-Collagenase and Pro-Stromelysin Exceeds Expression of Proteolytic Activity. *J. Biol. Chem.* **261**, 2810-2813.

Hinek A., Reiner A. & Poole A.R. (1987) The Calcification of Cartilage Matrix in Chondrocyte Culture: Studies of the C-propeptide of Type II Collagen (Chondrocalcin). *J. Cell Biol.* **104**, 1435-1441.

Hollander A. P., Heathfield T. F., Webber C., Iwata Y., Bourne R., Rorabeck C. & Poole A. R. (1994) Increased Damage to Type II Collagen in Osteoarthritic Articular Cartilage Detected by a New Immunoassay *J. Clin. Invest.* **93**, 1722-1732.

Hopkinson I., Evans W., Chant D., Hiscox S., Berry D & Harding K. (1995) Reverse Transcription-Polymerase Chain Reaction Detection of Collagen Transcripts in Healing Human Wounds *Eur. J. Clin. Invest.* **25**, 539-542.

Horton W. & Hassel J. R. (1986) Independence of Cell Shape and Loss of Cartilage Matrix Production During Retinoic Acid Treatment of Cultured Chondrocytes. *Dev. Biol.* **115**, 392-397.

Howell D. S., Sapolsky A. I., Pita J. C. & Woessner F. (1976) The Pathogenesis of Osteoarthritis. *Sem. Arth. Rheum.* **5**, 365-383.

Hoyland J. A., Thomas J. T., Donn R., Marriot A. Ayad S., Boot-Handford R. P., Grant M. E. & Freemont A. J. (1991) Distribution of Type X Collagen mRNA in Normal and Osteoarthritic Human Cartilage. *Bone & Mineral* **15**, 151-164.

Hulth A. (1993) Does Osteoarthritis Depend on Growth of the Mineralised Layer of Cartilage. *Clin. Orthop. Rel. Res.* **287**, 19-24.

Hwang W. S., Li B., Jin L. H., Ngo K., Schachar N. S. & Hugh G. N. F. (1992) Collagen Fibril Structure of Normal, Aging and Osteoarthritic Cartilage. *J. Path.* **167**, 425-433.

Ilic M. Z., Mok M. T., Williamson O. D., Campbell M. A., Hughes C. E. & Handley C. J. (1995) Catabolism of Aggrecan by Explant Cultures of Human Articular Cartilage in the Presence of Retinoic Acid. *Arch. Biochem. Biophys.* **322**, 22-30.

Iruela-Arispe M. L., Bornstein P. & Sage H. (1991) Thrombospondin Exerts an Antiangiogenic Effect on Cord Formation by Endothelial Cells *In-Vitro*. *Proc. Natl. Acad. Sci. USA* **88**, 5026-5030.

Ishizaki Y., Burne J. F. & Raff M. C. (1994) Autocrine Signals Enable Chondrocytes to Survive in Culture. *J. Cell Biol.* **126**, 1069-1077.

Iwamoto M., Shimazu A. & Pacifici M. (1995) Regulation of Chondrocyte Maturation by FGF-2 and Parathyroid Hormone. *J. Orthop. Res.* **13**, 838-845.

Iwamoto M., Yagami K., Shapiro I. M., Leboy P. S., Adams S. L. & Pacifici M. (1994) Retinoic Acid is a Major Regulator of Chondrocyte Maturation and Matrix Mineralisation. *Microscopy. Res. Technique* **28**, 483-491.

Iyama K., Ninomiya Y., Olsen B. R., Linsenmayer T. F., Trelstad R. L & Hayashi M. (1991) Spatiotemporal Pattern of Type X Collagen Gene Expression and Collagen Deposition in Embryonic Chick Vertebrae Undergoing Endochondral Ossification. *Anat. Rec.* **229**, 462-472.

Jacenko O., LuValle P. A. & Olsen B. R. (1993) Spodylometaphyseal Dysplasia in Mice Carrying a Dominant Negative Mutation in a Matrix Protein Specific for Cartilage-to-Bone Transition. *Nature* **365**, 56-61.

Jeffery A. K. (1973) Osteogenesis in the Osteoarthritic Femoral Head. A Study Using Radioactive $^{32}P$ and Tetracycline Bone Markers. *J. Bone Joint Surg.* **55B**, 262-272.

Jeffery A. K. (1994) Articular Cartilage and the Orthopaedic Surgeon. Part 1: Structure and Function. *Current Orthopaedics* **8**, 38-44.

Jeffery A. K., Blunn G. W., Archer C. W. & Bentley G. (1991) Three-Dimensional Collagen Architecture in Bovine Articular Cartilage. *J. Bone Joint Surg.[Br]* **73-B**, 795-801.

Kaji T., Kawatani R., Hoshino T., Miyahara T., Kozuka H., Kurashige Y. & Koizumi F. (1990) A suitable Culture Medium for Ossification of Embryonic Chick Femur in Organ Culture. *Bone & Mineral* **9**, 89-100.

Keilty C. M., Kwan A. P. L., Holmes D. F., Schor S. L. & Grant M. E. (1985) Type X Collagen, a Product of Hypertrophic Chondrocytes. *Biochem. J.* **227**, 545-554.

Kirsch T. & Pfaffle M. (1992) Selective Binding of Anchorin CII (Annexin V) to Type II and X Collagen and to Chondrocalcin (C-Propeptide of Type II Collagen). Implications for Anchoring Function Between Matrix Vesicles and Matrix Proteins. *FEBS Lett.* **310**, 143-147.

Kirsch T. & von der Mark K. (1991) Isolation of Human Type X Collagen and Immunolocalisation in Fetal Human Cartilage. *Eur. J. Biochem.* **196**, 575-580.

Kirsch T., Swoboda B. & von der Mark K. (1992) Ascorbate Independent Differentiation of Human Chondrocytes *In Vitro*: Simultaneous Expression of Types I and X Collagen and Matrix Mineralisation. *Differentiation* **52**, 89-100.

Kistler A., Galli B. & Kuhn H. (1991) Retinoic Acid-Induced Cartilage Degradation is Caused by Cartilage Cells. *Dev. Biol.* **199**, 377-386.

Kittelberger R., Davis P. F. & Greenhill N. S. (1989) Immunolocalisation of Type VIII Collagen in Vascular Tissue. *Biochem. Biophys. Res. Comm.* **159**, 414-419.

Kocher T. D. & Wilson A. C. (1991) DNA Amplification by the Polymerase Chain Reaction In: *Essential Molecular Biology: A Practical Approach, Volume II.* Ed. Brown T. A. Oxford University Press, pp.185-207.

Koopman G., Reutelingsperger C. P., Kuijten G. A., Keehnen R. M., Pals S. T. & van Oers M. H. (1994) Annexin V for Flow Cytomeric Detection of Phosphatidylserine Expression on B Cells Undergoing Apoptosis. *Blood* **84**, 1415-1420.

Korver G. H. V., Van de Stadt R. J., Van Kampen G. P. J. & Van der Korst J. K. (1990) Composition of Proteoglycans Synthesised in Different Layers of Cultured Anatomically Intact Articular Cartilage. *Matrix* **10**, 394-401.

Kosher R. A. & Solursh M. (1989) Widespread Distribution of Type II Collagen During Embryonic Chick Development. *Dev. Biol.* **131**, 558-566.

Kosher R. A. (1983) The Chondroblast and the Chondrocyte. In: *Cartilage* Ed. B. K. Hall. Volume 1: *Structure, Function and Biochemistry.* New York, Academic Press, pp59-85.

Kuettner K. E. (1992) Biochemistry of Articular Cartilage in Health and Disease. *Clinical Biochemistry* **25**, 155-161.

Kuhn (1987) The Classical Collagens: Types I, II and III. In: *Structure and Function of Collagen Types.* Eds. Mayne R. & Burgeson R. E. New York, Academic Press, pp 1-42. (181)

Kumar S., Kumar P., Pye D., Sattar A., Wang M. & Ponting J. (1992) Heterogeneity in Endothelial Cells with Special Reference to their Growth Related Proteins. In: *Angiogenesis in Health and Disease.* Ed. Maragoudakis M. E. Plenum Press pp 63-78.

Kwan A. P. L., Cummings C. E., Chapman J. A. & Grant M. E. (1991) Macromolecular Organisation of Chicken Type X Collagen *In-Vitro*. *J. Cell Biol.* **114**, 597-604.

Laemmli U. K. (1970) Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature* **227**, 680-685.

Lafeber F. P. J. G., van der Kraan P. M., van Roy J. L. A. M., Huber-Bruning & Bijlsma J. W. J. (1993) Articular Cartilage Explant Culture: An Appropriate *In Vitro* System to Compare Osteoarthritic and Normal Human Cartilage. *Connective Tissue Res.* **29**, 287-299.

Lafeber F.P.J.G., van der Kraan P. M., van Roy H. L. A. M., Vitters E. L., Huber Brunning O., van den Berg W. & Bijlsma J. W. J. (1992a) Local Changes in Proteoglycan Synthesis During Culture Are Different for Normal and Osteoarthritic Cartilage. *Am. J. Path.* **140**, 1421-1429.

Lafeber F. P. J. G., van Roy H., Wilbrink B., Huber-Bruning O. & Bijlsma J. W. J. (1992b) Human Osteoarthritic Cartilage is Synthetically More Active but in Culture Less Vital Than Normal Cartilage. *J. Rheumatol.* **19**, 123-129.

Lane J. M. & Weiss C. (1975) Review of Articular Cartilage Collagen Research. *Arth. Rheum.* **18**, 553-562.

Langille R. M. & Solursh M. (1990) Formation of Chondrous and Osseous Tissue in Micromass Cultures of Rat Frontonasal and Mandibular Ectomesenchyme. *Differentiation* **44**, 197-206.

Lark M. W., Bayne E. K., Flanagan J., Harper C. F., Hoerrner L. A., Hutchinson N. I., Singer I. I., Donatelli S. A., Weidner J. R., Williams H. R., Mumford R. A. & Lohmander L. S. (1997) Aggrecan Degradation in Human Cartilage. Evidence for Both Matrix Metalloproteinase and Aggrecanase Activity in Normal, Osteoarthritic and Rheumatoid Joints. *J. Clin. Invest.* **100**, 93-106.

Leboy P.S., Vaias L., Uschmann B., Golub E., Adams S. L. & Pacifici M. (1989) Ascorbic Acid Induces Alkaline Phosphatase, Type X Collagen and Calcium Deposition in Cultured Chick Chondrocytes. *J. Biol. Chem.* **264**, 17281-17286.

Leist M., Single B., Castoldi A. F., Kuhnle S. & Nicotera P. (1997) Intracellular Adenosine Triphosphate (ATP) Concentration: A Switch in the Decision Between Apoptosis and Necrosis. *J. Exp. Med.* **185**, 1481-1486.

Lewinson D. & Silbermann M. (1992) Chondroclasts and Endothelial Cells Collaborate in the Process of Cartilage Resorption. *Anat. Rec.* **233**, 504-514.

Linsenmayer T. F. (1991) Collagen. In: *Cell Biology of Extracellular Matrix, Second Edition* .Ed. Hay E. D. Plenum Press, New York. pp7-43.

Lipiello L., Hall M. D. & Mankin H. J. (1977) Collagen Synthesis in Normal and Osteoarthritic Human Cartilage. *J. Clin. Invest.* **59**, 593-600.

Lohmander L. S. (1994) Articular Cartilage and Osteoarthrosis. The Role of Molecular Markers to Monitor Breakdown, Repair and Disease. *J. Anat.* **184**, 477-492.

Lohmander L. S., Neame P. J. & Sandy J. D. (1993) The Structure of Aggrecan Fragments in Human Synovial Fluid. Evidence that Aggrecanase Mediates Cartilage Degradation in Inflammatory Joint Disease, Joint Injury and Osteoarthritis. *Arth. Rheum.* **36**, 1214-1222.

Lotz M., Blanco F. J., von Kempis J, Dudler J., Maier R., Villiger P. M. & Geng Y. (1995) Cytokine Regulation of Chondrocyte Functions. *J. Rheumatol.* **22**, 104-108.

MacConaill M. A. (1951) The Movements of Bones and Joints: 4. The Mechanical Structure of Articulating Cartilage. *J. Bone Joint Surg.[Br]* **33-B**, 251-257.

Mallein-Gerin F., Garrone R. & van der Rest M. (1991) Proteoglycan and Collagen Synthesis are Correlated with Actin Organisation in Dedifferentiating Chondrocytes. *Eur. J. Cell Biol.* **56**, 364-373.

Mallein-Gerin F., Ruggiero F. & Garrone R. (1990) Proteoglycan Core Protein and Type II Collagen Gene Expression are not Correlated with Cell Shape Changes During Low Density Chondrocyte Cultures. *Differentiation* **43**, 204-211.

Mallein-Gerin F., Ruggiero F., Quinn T. M., Bard F., Grodzinsky A. J., Olsen B. R. & van der Rest M. (1995) Analysis of Collagen Synthesis and Assembly in Culture by Immortalised Mouse Chondrocytes in the Presence or Absence of α1(IX) Collagen Chains. *Exp. Cell Res.* **219**, 257-265.

Mankin H. J. (1974a) The Reaction of Articular Cartilage to Injury and Osteoarthritis (Part I). *New. Engl. J. Med.* **291**, 1285-1292.

Mankin H. J. (1974b) The Reaction of Articular Cartilage to Injury and Osteoarthritis (Part II). *New. Engl. J. Med.* **291**, 1335-1340.

Mankin H. J., Dorfman H., Lippiello L. & Zarins A. (1971) Biochemical and Metabolic Abnormalities in Articular Cartilage from Osteoarthritic Human Hips. (II. Correlation of Morphology with Biochemical and Metabolic Data). *J. Bone Joint Surg.* **53A**, 523-537.

Mankin H. J., Johnson M. E. & Lippiello L. (1981) Biochemical and Metabolic Abnormalities in Articular Cartilage from Osteoarthritic Human Hips. (III) Distribution and Metabolism of Amino Sugar-Containing Macromolecules. *J. Bone Joint Surg.* **63A**, 131-139.

Maroudas A., Ziv I., Weisman N. & Venn M. (1985) Studies of Hydration and Swelling Pressure in Normal and Osteoarthritic Cartilage. *Biorheology* **22**, 159-169.

Martel-Pelletier J., Zafarullah M., Kodama S. & Pelletier J.-P. (1991) *In Vitro* Effects of Interleukin 1 on the Synthesis of Metalloproteases, TIMP, Plasminogen Activators and Inhibitors in Human Articular Cartilage. *J. Rheumatol.* **18** (Supp. 27), 80-84.

Martin S. J., Reutelingsperger C. P., McGahon A. J., Rader J. A., van Schie R. C., LaFace D. M. & Green D. R. (1995) Early Redistribution of Plasma Membrane Phosphatidylserine is a General Feature of Apoptosis Regardless of the Initiating Stimulus: Inhibition by Overexpression of Bcl-2 and Abl. *J.Exp. Med.* **182,** 1545-1556.

Matrisian L. M. (1990) Metalloproteinases and their Inhibitors in Matrix Remodelling. *Trends in Genetics* **6**, 121-125.

Mayne R. (1989) Cartilage Collagens. What is their Function, and are they Involved in Artricular Disease? *Arth. Rheum.* **32**, 241-246.

Mayne R., Vail M. S., Mayne P. M. & Miller E. J. (1976) Changes in the Type of Collagen Synthesised as Clones of Chick Chondrocytes Grow and Eventually Lose Division Capacity. *Proc. Natl. Acad. Sci. USA* **73**, 1674-1678.

McCollum R., Martel-Pelletier J., DiBattista J. & Pelletier J.-P. (1991) Regulation of Interleukin 1 Receptors in Human Articular Chondrocytes. *J. Rheumatol.* **18** (Supp 27), 85-88.

Meachim G. (1972) Articular Cartilage Lesions in Osteoarthritis of the Femoral Head. *J. Path.* **107**, 199-210.

Mendler M., Eich-Bender S. G., Vaughan L., Winterhalter K. H. & Bruckner P. (1989) Cartilage Contains Mixed Fibrils of Collagen Types II, IX and XI. *J. Cell Biol.* **108**, 191-198.

Miller E. J. & Matukas V.J. (1969) Chick Cartilage Collagen: A New Type of α1 Chain Not Present in Bone or Skin of the Species. *Proc. Natl. Acad. Sci. USA* **64**, 1264-1268.

Mort J. S., Dodge G. R., Roughley P. J., Liu J., Finch S. J., DiPasquale G. & Poole A. R. (1993) Direct Evidence for Active Metalloproteinases Mediating Matrix Degradation in Interleukin-1 Stimulated Human Articular Cartilage. *Matrix* **13**, 95-102.

Moses M. A. (1993) A Cartilage Derived Inhibitor of Neovascularisation and Metalloproteinases. *Clin. Exp. Rheumatol.* **11**(suppl 8), S67-S69.

Moses M. A., Sudhalter J. & Langer R. (1990) Identification of an Inhibitor of Neovascularisation from Cartilage. *Science* **248**, 1408-1410.

Nakata K., Nakahara H., Kimura T., Kojima A., Iwasaki M., Caplan A. I. & Ono K. (1992) Collagen Gene Expression During Chondrogenesis from Chick Periosteum Derived Cells. *FEBS Lett.* **299**, 278-282.

Nerlich A. G., Wiest I. & von der Mark K. (1993) Immunohistochemical Analysis of Interstitial Collagens in Cartilage of Different Stages of Osteoarthritis. *Virchows Archiv B Cell Pathol.* **63**, 249-255.

Nguyen M., Shing Y. & Folkman J. (1994) Quantification of Angiogenesis and Anti-angiogenesis in the Chick Embryo Chorio-allantoic Membrane. *Microvascular Res.* **47**, 31-40.

Nimni M. & Deshmukh K. (1973) Differences in Collagen Metabolism Between Normal and Osteoarthritic Cartilage. *Science* **181**, 751-752.

O'Keefe R. J., Loveys L. S., Hicks D. G., Reynolds P. R., Crabb I. D., Puzas J. E. & Rosier R. N. (1997) Differential Regulation of Type-II and Type-X Collagen Synthesis by Parathyroid Hormone-Related Protein in Chick Growth-Plate Chondrocytes. *J. Orthop. Res.* **15**, 162-174.

Olsen B. R. (1995) Mutations in Collagen Genes Resulting in Metaphyseal and Epiphyseal Dysplasias. *Bone* **17**, 45S-49S.

Pacifici M., Golden E. B., Adams S. L. & Shapiro I. M. (1991) Cell Hypertrophy and Type X Collagen Synthesis in Cultured Articular Chondrocytes. *Exp. Cell Res.* **192**, 266-270.

Pacifici M., Golden E. B., Oshima O., Shapiro I. M., Leboy P.S. & Adams S. L. (1990) Hypertrophic Chondrocytes. The Terminal Stage of Differentiation in the Chondrogenic Cell Lineage. *Ann. New York Acad. Sci.* **599**, 45-57.

Paulson D. F., Chen W. D., Pang L. Johnson B. & Okello D. (1994) Stage and Region Dependent Chondrogenesis and Growth of Chick Wing Bud Mesenchyme in Serum Containing and Defined Tissue Culture Media. *Dev. Dynam.* **200**, 39-52.

Pechak D. G., Kujawa M. J. & Caplan A. I. (1986a) Morphological and Histochemical Events During First Bone Formation in Embryonic Chick Limbs. *Bone* 7, 441-458.

Pechak D. G., Kujawa M. J. & Caplan A. I. (1986b). Morphology of Bone Development and Bone Remodelling in Embryonic Chick Limbs. *Bone* 7, 459-472.

Pelletier J.-P., DiBattista J. A., Roughley P., McCollum R. & Martell-Pelletier J. (1993) Cytokines and Inflammation in Cartilage Degradation. *Rheum. Dis. Clin. N. Am.* **19**, 545-568.

Pelletier J.-P., Martell-Pelletier J., Howell D. S., Ghandurmnaymneh L., Enis J. E. & Woessner J. F. (1983) Collagenase and Collagenolytic Activity in Human Osteoarthritic Cartilage. *Arth. Rheum.* **26**, 63-68.

Pelletier J.-P., Mineau F., Faure M. P. & Martell-Pelletier J. (1990) Imbalance Between the Mechanisms of Activation and Inhibition (TIMP) of Metalloproteases in the Early Lesions of Experimental Osteoarthritis. *Arth. Rheum.* **33**, 1466-1476.

Peltonen L. (1990) New Views to the Molecular Pathology of Osteoarthrosis. *Ann. Med.* **22**, 219-220.

Pepper M. S., Montesano R. Vassalli J-D. & Orci L. (1991) Chondrocytes Inhibit Endothelial Sprout Formation *In-Vitro*: Evidence for Involvement of a Transforming Growth Factor-Beta. *J. Cell. Physiol.* **146**, 170-179.

Poole A. R., Rizkalla G., Ionescu M., Reiner A., Brooks E., Rorabeck C., Bourne R. & Bogoch E. (1993) Osteoarthritis in the Human Knee: A Dynamic Process of Cartilage Matrix Degradation, Synthesis and Reorganisation. In: *Joint Destruction in Arthritis and Osteoarthritis*. Ed. Van Den Berg W. W. *et al.* Berghaus Verlag, Basal. pp3-13.

Poole C. A., Ayad S. & Schofield J. R. (1988) Chondrons from Articular Cartilage. 1. Immunolocalisation of Type VI Collagen in the Pericellular Capsule of Isolated Canine Tibial Chondrons. *J. Cell Sci.* **90**, 635-643.

Posever J., Phillips F. M. & Lawrence P. A. (1995) Effects of Basic Fibroblast Growth Factor, Transforming Growth Factor-β1, Insulin-like Growth Factor-1, and Insulin on Human Osteoarthritic Articular Cartilage Explants. *J. Bone Joint Surg.* **13A**, 832-837.

Price J. S., Oyajobi B. O. & Russell R. G. G. (1994) The Cell Biology of Bone Growth. *Eur. J. Clin. Nutrition* **48** (Suppl. 1), S131-S149.

Pujol J.-P., Galera P., Redini F., Mauviel A. & Loyau G. (1991) Role of Cytokines in Osteoarthritis: Comparative Effects of Interleukin 1 and Transforming Growth Factor-β on Cultured Rabbit Articular Chondrocytes. *J. Rheumatol.* **18** (Supp. 27), 76-79.

Rao L. V., Tait J. F. & Hoang A. D. (1992) Binding of Annexin V to a Human Ovarian Carcinoma Cell Line (OC-2008). Contrasting Effects on Cell Surface Factor VIIa/Tissue Factor Activity and Prothrombinase Activity. *Thrombosis Res.* **67**, 517-531.

Rayan V. & Hardingham T. (1994) The Recovery of Articular Cartilage in Explant Culture from Interleukin-1α: Effects on Proteoglycan Synthesis and Degradation. *Matrix Biol.* **14**, 263-271.

Reboul P., Pelletier J. P., Tardif G., Cloutier J.-M. & Martell-Pelletier J. (1996) The New Collagenase, Collagenase-3, is Expressed and Synthesised by Human Chondrocytes but not by Synoviocytes. A Role in Osteoarthritis. *J. Clin. Invest.* **97**, 2011-2019.

Reddi A. H. & Kuettner K.E. (1981) Vascular Invasion of Cartilage: Correlation of Morphology with Lysozyme, Glycosaminoglycans, Protease and Protease Inhibitory Activity During Endochondral Bone Development. *Dev. Biol.* **82**, 217-223.

Reginato A. M., Tuan R. S., Ono T., Jimenez S. A. & Jacenko O. (1993) Effects of Calcium Deficiency on Chondrocyte Hypertrophy and Type X Collagen Expression in Chick Embryonic Sternum. *Dev. Dynam.* **198**, 284-295.

Reichenberger E., Aigner T., von der Mark K. & Bertling W. (1991) *In-Situ* Hybridisation Studies on the Expression of Type X Collagen in Fetal Human Cartilage. *Dev. Biol.* **148**, 562-572.

Repo R. U. & Mitchell N. S. (1971) Collagen Biosynthesis in Mature Articular Cartilage of the Rabbit. *J. Bone Joint Surg.* **53B**, 541-548.

Roach H. I. & Shearer J. R. (1989) Cartilage Resorption and Endochondral Bone Formation During the Development of Long Bones in Chick Embryos. *Bone and Mineral* **6**, 289-309.

Roach H. I. (1992) Trans-Differentiation of Hypertrophic Chondrocytes into Cells Capable of Producing Mineralised Bone Matrix. *Bone & Mineral* **19**, 1-20.

Roach H. I., Erenpreisa J. & Aigner T. (1995) Osteogenic Differentiation of Hypertrophic Chondrocytes Involves Asymmetric Cell Divisions and Apoptosis. *J. Cell Biol.* **131**, 483-494.

Ronziere M-C., Ricard-Blum S., Tiollier J., Hartmann D. J. Garrone R. & Herbage D. (1990) Comparitive Analysis of Collagens Solubilised from Human Foetal, and Normal and Osteoarthritic Adult Articular Cartilage, with Emphasis on Type VI Collagen. *Biochim. Biophys. Acta* **1038**, 222-230.

Rooney P. & Archer C. W. (1992) The Development of the Perichondrium in the Avian Ulna. *J. Anat.* **181**, 393-401.

Rooney P., Archer C. & Wolpert L. (1984) Morphogenesis of Cartilaginous Long Bone Rudiments. In: *The Role of Extracellular Matrix in Development.* Ed. Trelstad R. L. New York: Alan R. Liss. pp305-322.

Rooney P. & Kumar S. (1993) Inverse Relationship Between Hyaluronan and Collagens in Development. *Differentiation* **54**, 1-9.

Rooney P., Kumar S., Ponting J. & Wang M. (1995) Role of Hyaluronan in Tumour Neovascularisation. *Int. J. Cancer* **60**, 632-636.

Rooney P., Kumar P., Ponting J. & Kumar S. (1997) Role of Collagen and Proteoglycan in Tumour Angiogenesis. In: *Tumour Angiogenesis.* Eds. Bicknell R., Lewis C. E. & Ferrara N. Oxford University Press pp141-151.

Rooney P., Wang M., Kumar P. & Kumar S. (1993) Angiogenic Oligosaccharides of Hyaluronan Enhance the Production of Collagen by Endothelial Cells. *J. Cell Sci.* **105**, 213-218.

Rooney P. (1984) The Cellular Basis of Cartilage Morphogenesis. Ph.D Thesis, University of London.

Rosati R., Horan G. S. B., Pinero G. J., Garofalo S., Keene D. R., Horton W. A., Vuorio E., de Crombrugghe B. & Behringer R. B. (1994) Normal Long Bone Growth and Development in Type X Collagen Null Mice. *Nature Genetics* **8**, 129-135.

Ryu J., Treadwell B. V. & Mankin H. J. (1984) Biochemical and Metabolic Abnormalities in Normal and Osteoarthritic Human Articular Cartilage. *Arth. Rheum.* **27**, 49-57.

Sambrook J., Fritsch E. F. & Maniatis T. (1989) *In Vitro* Amplification of DNA by the Polymerase Chain Reaction. In; *Molecular Cloning: A Laboratory Manual.* Ed. Nolan C. Cold Spring Harbour Laboratory Press pp.14.1-14.35.

Sandell L. J., Sugai J. V. & Trippel S. B. (1994) Expression of Collagens I, II, X and XI and Aggrecan mRNAs by Bovine Growth Plate Chondrocytes *In Situ.* *J. Orthop. Res.* **12**, 1-14.

Schmid T. M. & Linsenmayer T. F. (1987) Type X Collagen. In: *Structure and Function of Collagen Types.* Eds. Mayne R. & Burgeson R. E. New York, Academic Press, pp 223-259.

Schmid T. M., Bonen D. K., Luchene L., Linsenmayer T. F. (1991) Late Events in Chondrocyte Differentiation: Hypertrophy, Type X Collagen Synthesis and Matrix Calcification. *In Vivo* **5**, 553-540.

Schmid T. M., Popp R. G. & Linsenmayer T. F. (1990) Hypertrophic Cartilage Matrix. Type X Collagen: Supramolecular Assembly and Calcification. *Ann. New York Acad. Sci.* **580**, 64-73.

Schor A. M., Canfield A. E., Sutton A. B., Arciniegas E. & Allen T. D. (1995) Pericyte Differentiation. *Clin. Orthop. & Rel. Res.* **313**, 81-91.

Schumacher B. L., Block J. A., Schmid T. M., Aydelotte M. B. & Kuettner K. E. (1994) A Novel Proteoglycan Synthesised and Secreted by Chondrocytes of the Superficial Zone of Articular Cartilage. *Archives Biochem. and Biophys.* **311**, 144-152.

Scott C. K. & Hightower J. A. (1991) The Matrix of Endochondral Bone Differs from the Matrix of Intramembranous Bone. *Calcif. Tissue Int.* **49**, 349-354.

Scott J. E. & Dorling J. (1965) Differential Staining of Acid Glycosaminoglycans (Mucopolysaccharides) by Alcian Blue in Salt Solutions. *Histochemie* **5**, 221-233.

Shingu M., Nagai Y., Isayama T., Naono T., Nobunaga M. & Nagai Y. (1993) The Effects of Cytokines on Metalloproteinase Inhibitors (TIMP) and Collagenase Production by Human Chondrocytes and TIMP Production by Synovial Cells and Endothelial Cells. *Clin. Exp. Immunol.* **94**, 145-149.

Silvestrini G., Ricordi M. E. & Bonucci E. (1979) Resorption of Uncalcified Cartilage in the Diaphysis of the Chick Embryo Tibia. *Cell Tiss. Res.* **196**, 221-235.

Skoloff L. (1987) Osteoarthritis as a Remodelling Process. *J. Rheumatol.* **14** (suppl. 1), 7-10.

Smith G. N.,Jr., & Brandt K. D. (1992) Hypothesis: Can Type IX Collagen "Glue" Together Intersecting Type II Fibers in Articular Cartilage Matrix? A Proposed Mechanism. *J. Rheumatol.* **19**,14-17.

Smith G. N.,Jr., Linsenmayer T. F. & Newsome D. A. (1976) Synthesis of Type II Collagen *in-vitro* by Embryonic Chick Neural Retina Tissue. *Proc. Natl. Acad. Sci. USA* **73**, 4420-4423.

Soames R. W. (1995) Skeletal System. In: *Grays Anatomy 38th Edition.* Eds. Williams P. L. *et al.* Churchill Livingstone. pp425-712.

Solursh M. & Reiter R. S. (1975) Determination of Limb Bud Chondrocytes During a Transient Block of the Cell Cycle. *Cell Diff.* **4**, 131-137.

Solursh M. (1989) Cartilage Stem Cells: Regulation of Differentiation. *Conn. Tiss. Res.* **20**, 81-89.

Solursh M., Linsenmayer T. F. & Jensen K. L. (1982) Chondrogenesis from Single Limb Mesenchyme Cells. *Dev. Biol.* **94**, 259-264.

Sorgente N., Kuettner K. E., Soble L. W. & Einstein R. (1975) The Resistance of Certain Tissues to Invasion. II. Evidence for Extractable Factors in Cartilage Which Inhibit Invasion by Vascularised Mesenchyme. *Lab. Invest.* **32**, 317-322.

Spotila L. D., Colige A., Sereda L., Constantinou-Deltas C. D., Whyte M. P., Olsen N., Attie M., Tennehouse A., Shane E., Briney W. & Prockop D. (1994) Mutation Analysis of Coding Sequences for Type I Procollagen in Individuals with Low Bone Density. *J. Bone Mineral Res.* **9**, 923-932.

Stephens M., Kwan A. P. L., Bayliss M. T. & Archer C. W. (1992) Human Articular Surface Chondrocytes Initiate Alkaline Phoaphatase and Type X Collagen Synthesis in Suspension Culture. *J. Cell Sci.* **103**, 1111-1116.

Sullivan T. A., Uschmann B., Hough R & Leboy P. S. (1994) Ascorbate Modulation of Chondrocyte Gene Expression is Independent of its Role in Collagen Secretion. *J. Biol. Chem.* **269**, 22500-22506.

Tada K., Fukunaga T., Wakabayashi Y., Masumi S., Sato Y., Izumi H., Kohno K. & Kuwano M. (1994) Inhibition of Tubular Morphogenesis in Human Microvascular Endothelial Cells by Co-Culture with Chondrocytes and Involvement of Transforming Growth Factor β: A Model for Avascularity in Human Cartilage. *Biochim. Biophys. Acta* **1201**, 135-142.

Teshima R., Otsuka T., Takasu N., Yamagata N. & Yamamoto K. (1995) Structure of the Most Superficial Layer of Articular Cartilage. *J. Bone Joint Surg.* **77B**, 460-464.

Testa V., Capasso G., Maffulli N., Sgambato A. & Ames R. J. (1994) Proteases and Antiproteases in Cartilage Homeostasis. *Clin. Orthop. Rel. Res.* **308**, 79-84.

Thomas J. T., Boot-Handford R. P. & Grant M. E. (1990) Modulation of Type X Collagen Gene Expression by Calcium β-Glycerophosphate and Levisamole - Implications for a Possible Role For Type X Collagen in Endochondral Bone Formation. *J. Cell Sci.* **95**, 639-648.

Thorogood P. (1983) The Morphogenesis of Cartilage. In: *Cartilage,* Vol. 2. Ed Hall B. K. New York : Academic Press. pp223-254.

Thorogood P. V. & Hinchcliffe J. R. (1975) An Analysis of the Condensation Process During Chondrogenesis in the Embryonic Chick Hind Limb. *J. Embryol. Exp. Morphol.* **33**, 581-606.

Timpl R. & Engle J. (1987) Type VI Collagen. In: *Structure and Function of Collagen Types.* Eds. Mayne R. & Burgeson R. E. New York: Academic Press, pp 105-143.

Towbin H., Staehelin T. & Gordon J. (1979) Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets: Procedure and Some Applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

Treadwell B. V. & Mankin H. J. (1986) The Synthetic Process of Articular Cartilage. *Clin. Orthop. Relat. Res.* **213**, 50-61.

Trippel S. B. (1990) Articular Cartilage Research. *Curr. Opinion Rheumatol.* **2**, 777-782.

Umansky R. (1966) The Effect of Cell Population Density on the Developmental Fate of Reaggregating Mouse Limb Bud Mesenchyme. *Dev. Biol.* **13**, 31-56.

van Beuningen H. M., van der Kraan P. M., Arntz O. J. & van den Berg W. B. (1993) Protection from Interleukin-1 Induced Destruction of Articular Cartilage by Transforming Growth Factor β: Studies in Anatomically Intact Cartilage *In Vitro* and *In Vivo*. *Ann. Rheum. Dis.* **52**, 185-191.

van de Loo F. A. J., Joosten L. A. B., Van Lent P. L. E. M., Arntz O. J. & van den Berg W. B. (1995) Role of Interleukin-1, Tumour Necrosis Factor α and Interleukin-6 in Cartilage Proteoglycan Metabolism and Destruction. *Arth. Rheum.* **38**, 164-172.

van Engeland M., Ramaekers F. C., Schutte B. & Reutelingsperger C. P. (1996) A Novel Assay to Measure Loss of Plasma Membrane Asymmetry During Apoptosis of Adherent Cells in Culture. *Cytometry* **24**, 131-139.

van-der Rest M. & Garrone R. (1991) Collagen Family of Proteins. *FASEB J.* **5**, 2814-2823.

Vaughan L., Mendler M., Huber S., Bruckner P., Winterhalter K., Irwin M. H. & Mayne R. (1988) D-periodic Distribution of Collagen Type IX Along Cartilage Fibrils. *J. Cell Biol.* **106**, 991-997.

Venn G., Billingham M. E. J. & Hardingham (1995) Increased Proteoglycan Synthesis in Cartilage in Experimental Canine Osteoarthritis Does Not Reflect a Permanent Change in Chondrocyte Phenotype. *Arth. Rheum.* **38**, 525-532.

Venn M. & Maroudas A. (1977) Chemical Composition and Swelling of Normal and Osteoarthritic Femoral Head Cartilage. I. Chemical Composition. *Ann. Rheum. Dis.* **36**, 121-129.

Vermes I., Haanen C., Steffens-Nakken H. & Reuteingsperger C. (1995) A Novel Assay for Apoptosis. Flow Cytometric Detection of Phosphatidylserine Expression on Early Apoptotic Cells Using Fluorescein labelled Annexin V. *J. Immunol Methods* **184**, 39-51.

von der Mark K., Kirsch T., Nerlich A., Kuss A., Weseloh G., Gluckert K. & Stoss H. (1992) Type X Collagen Synthesis in Human Osteoarthritic Cartilage . Indication of Chondrocyte Hypertrophy. *Arth. Rheum.* **35**, 806-811.

Vortkamp A. S., Lee K., Lanske B., Segre G. V., Kronenberg H. M. & Tabin C. J. (1996) Regulation of Cartilage Differentiation by Indian Hedgehog and PTH-Related Proteins. *Science*, **273**, 613-622.

Wallis G. A. (1993) Here today, bone tomorrow.*Current Biology* **3**, 687-689.

Wight T. N., Heinegard D. K. & Hascall V. C. (1991) Proteoglycans. In: *Cell Biology of Extracellular Matrix, Second Edition.* Ed. Hay E.D. Plenum Press, New York, pp45-78.

Woessner Jr. J. F. & Gunja Smith Z. (1991) Role of Metalloproteinases in Human Osteoarthritis. *J. Rheumatol.* **18**(suppl 27), 99-101.

Wolfe J. T., Pringle J. H. & Cohen G. M. (1996) Measurement of DNA Fragmentation During Apoptosis. In: *Techniques in Apoptosis: A Users Guide.* Eds. Cotter T. G. & Martin S. J. Portland Press. pp. 51-70.

Wolpert L. (1982) Cartilage Morphogenesis in the Limb. In: *Cell Behaviour.* Eds. Bellairs R., Curtis A. S. G., Dunn G. A. Cambridge: Cambridge University Press, pp 359-372.

Wu J. J. & Eyre D. R. (1989) Covalent Interactions of Type IX Collagen in Cartilage. *Conn. Tiss. Res.* **20**, 241-246.

Yamada N., Nakagawa T., Stephens R. W. & Nagai Y. (1987) Proteinase and their Inhibitors in Normal and Osteoarthritic Articular Cartilage. *Biomed. Res.* **8**, 289-300.

Zenmyo M., Komiya S., Kawabata R., Sasaguri Y., Inoue A. & Morimatsu M. (1996) Morphological and Biochemical Evidence for Apoptosis in the Terminal Hypertrophic Chondrocytes of the Growth Plate. *J. Pathol.* **180**, 430-433.

Zimmermann B., Lange K., Mertens P. & Bernimoulin J.-P. (1994) Inhibition of Chondrogenesis and Endochondral Mineralisation *In Vitro* by Different Calcium Channel Blockers. *Eur. J. Cell Biology* **63**, 114-121.