ANALYSIS OF PHYSIOLOGICAL DEATH IN EQUINE CHONDROCYTES

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ABSTRACT

Chondrocytes in growth cartilage undergo proliferation, hypertrophy, and then die by a mechanism that has not been characterised. The aims of the current study were to document the morphology of dying hypertrophic chondrocytes in equine growth cartilage and to establish a culture system in which the isolated chondrocytes can be induced to undergo the same modes of hypertrophy and physiological death seen in growth cartilage in vivo.

Growth cartilage from foetal and growing postnatal horses was examined by electron microscopy. Ultrastructural studies of the tissue specimens suggested that the two types of hypertrophic chondrocytes that have previously been described as dark and light cells were dying by different non-apoptotic forms of cell death. Dying hypertrophic dark chondrocytes were characterised by a dark nucleus, and their cytoplasm appeared to undergo extrusion into the extracellular matrix, whereas light chondrocytes appeared to disintegrate within the cell membrane.

Chondrocytes were isolated from growth cartilage of foetal, neonatal and older (growing and adult) horses, and cultured as pellets in either 10% foetal calf serum or 10% horse serum, then were processed for light and electron microscopy. Foetal chondrocytes in either serum type underwent dark and light hypertrophic differentiation and were dying by the same modes of physiological cell death observed in growth cartilage in vivo. Chondrocyte pellet cultures from neonatal and older horses cultured in 10% foetal calf serum did not undergo hypertrophy, but they contained inclusion bodies and some cells were similar to osteoblasts. In 10% horse serum, chondrocytes from neonatal foals differentiated into hypertrophic dark and light chondrocytes but also contained inclusion bodies, and chondrocytes from older horses were similar to those grown in 10% FCS. These results indicated that foetal samples are more suitable for induction of chondrocyte hypertrophy and death in the chondrocyte pellet culture system.

To investigate the possibility of manipulation of the proportion of different cell types in cultures, pellets were cultured in either 10% foetal calf serum or 0.1% foetal calf serum in the presence or absence of triiodothyronine or transforming growth factor-β1. Addition of transforming growth factor-β1 and 10% foetal calf serum to
pellet culture increased the proportion of dark chondrocytes and induced morphologically typical dark cell death, however, triiodothyronine increased dark and light cell differentiation and induced their death. From these studies, it was determined that 10% FCS provided the best conditions for obtaining hypertrophic dark cells and inducing their death, and that T3 in 0.1% FCS provided the best conditions for obtaining pellets containing hypertrophic light cells and inducing their death.

Total RNA was extracted from pellets with a high proportion of specific forms of chondrocytes in early and dying stages and used for quantitative polymerase chain reaction studies. Dark cell-enriched pellets showed significantly higher levels of expression of Sox9, metalloproteinase-13, vascular endothelial growth factor, connective tissue growth factor and collagen type I mRNA than light cell-enriched pellets while light cell-enriched pellets showed significantly higher levels of expression of collagen type II and Runx2 mRNA than dark cell-enriched pellets. No significant difference in aggrecan and fibroblast growth factor receptor-3 mRNA expression was found between the two cell types. These studies indicated that hypertrophic dark and light chondrocytes differ in their patterns of gene expression.

From the observations presented in this thesis it was concluded that dark and light hypertrophic chondrocytes appear to be different cell populations with different morphology and gene expression. These cells were shown to die by non-apoptotic physiological modes of death. It has been demonstrated that dark and light chondrocyte differentiation as well as death can be replicated in vitro by varying the culture conditions. The culture system will be useful for future investigation of factors regulating dark and light cell death as well as the molecular mechanisms by which dark and light chondrocytes die.
DECLARATION

This is to certify that

i. the thesis comprises only my original work towards the PhD

ii. due acknowledgment has been made in the text to all other material used

iii. the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Yasser Abdel Galil Ahmed

ABSTRACTS PRESENTED DURING THE CANDIDATURE OF THIS THESIS


- Ahmed YA, Tatarczuch L, Chen K-S, Pagel CN, Davies HM, Mirams M, Mackie EJ. Establishment of an in vitro model for studying physiological


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**LIST OF ABBREVIATIONS**

3-D ............................................................................................. Three-dimensional  
ABC ............................................................................................. Avidin-biotin complex  
AEGC ..................................................................................... Articular epiphyseal growth cartilage  
ATP ................................................................................... Adenosine tri-phosphate  
BMP ................................................................................... Bone morphogenic proteins  
BSA ...................................................................................... Bovine serum albumin  
cDNA ..................................................................................... Complementary DNA  
CT ................................................................................................... Cycle threshold  
CTGF ........................................................................... Connective tissue growth factor  
DAB ................................................................................... Diaminobenzidine tetrahydrochloride  
DAPI ........................................................................ 4,6-diamidino-2-phenylindole  
DEPC .................................................................................... Diethylpyrocarbonate  
DMEM ........................................................ Dulbecco's Modified Eagle’s Medium  
DMSO....................................................................................... Dimethyl sulphoxide  
dNTPs (dATP, dCTP, dGTP, dTTP) ... Deoxynucleotide triphosphates (A-adenine, C-cytosine, G-guanine, T-thymidine)  
DPX ................................................................................... Distyrene dibutylphthalate and xylene  
ECM ......................................................................................... Extracellular matrix  
EDTA ........................................................................... Ethylene-diamine-tetra-acetic acid  
FCS ................................................................................................ Foetal calf serum  
FGF ..................................................................................... Fibroblast growth factor  
FGFR ................................................................................ Fibroblast growth factor receptor  
HGF ................................................................................... Hepatocyte growth factor  
HS ............................................................................................ Horse serum  
IGF ................................................................................... Insulin-like growth factor  
Ihh ................................................................................................... Indian hedgehog  
IL-1β ............................................................................................. Interleukin-1β  
LEL ................................................................................... Lamina-enclosed light cell  
M-CSF ................................................................................ Macrophage-Colony Stimulating factor  
M-MLV ................................................................................ Moloney Murine Leukemia Virus
MMP .......................................................... Matrix metalloproteinase
PBS ........................................................... Phosphate-buffered saline
PCD .............................................................. Physiological cell death
PCR .............................................................. Polymerase chain reaction
PDGF ........................................................ Platelet-derived growth factor
PGC .............................................................. Physeal growth cartilage
POC .............................................................. Primary ossification centre
PTH .............................................................. Parathyroid hormone
PTHrP ...................................................... parathyroid hormone related peptide
Q-PCR ........................................................ Quantitative real time PCR
RANKL ........................................ Receptor activator for nuclear factor κ B Ligand
RBCs .......................................................... Red blood cells
RER .............................................................. Rough endoplasmic reticulum
RP-S23 ....................................................... Ribosomal protein S23
RT ................................................................. Reverse transcription
SDS ............................................................ Sodium dodecyl sulphate
SOC ............................................................. Secondary ossification centre
T3 ............................................................... Tri-iodothyronine
TBE ........................................................... Tris base, boric acid and EDTA
TESPA ......................................................... 3-aminopropyltriethoxysilane
TGF-β ......................................................... Transforming growth factor- β
TUNEL ....................................................... Deoxynucleotidyl transferase-mediated dUTP nick-end labeling
VEGF ........................................................ Vascular endothelial growth factor

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CHAPTER 1: LITERATURE REVIEW

1.1 Skeletal Development and Growth

1.1.1 Bone and Cartilage Structure and Function

The main role of the skeleton is to support other tissues and provide levers for locomotion. The tissues of the skeleton form part of the connective tissue of the animal body. The connective tissue is formed generally of cells and extracellular matrix (ECM). Depending on the types of cells and the contents of ECM, connective tissue is classified into many types. For example, adipose connective tissue contains mostly fat cells and acts as cushions to protect vital organs such as the kidney. Lymphatic tissue and blood are also considered as types of connective tissue. Dense connective tissue is formed mainly of fibroblasts and collagen fibres. Cartilage and bone are two types of hard connective tissue that contain highly metabolically active specialised cells, chondrocytes in cartilage, and osteoblasts and osteoclasts in bone (Fig. 2.1, Junqueira et al., 1998).

1.1.1.1 Bone

Bone tissue is the major component of the adult body and carries out vital functions for the animal. It supports body weight and protects organs such as the brain, heart and lung. Bones articulate with each other to allow the movements of the skeleton, and provide surfaces for muscle and ligament attachment. Not only does bone tissue fulfil these mechanical and structural functions but it also plays an important role in maintenance of physiological function. Bone is the largest store of calcium and phosphorus, the minerals that give bones their characteristic rigidity (Junqueira et al., 1998). In addition, bone marrow is the main source of the different blood cells.

Long bone consists of two ends (epiphyses) and a shaft (diaphysis). The diaphysis is separated from the epiphyses by a narrow zone (metaphysis; Fig. 1.1A). The diaphysis is composed mostly of compact (cortical) bone and contains the medullary cavity, which houses the bone marrow. The epiphysis and metaphysis are composed
largely of spongy (trabecular) bone. Long bone has two surfaces, the external surface (periosteum) and internal surface (endosteum; Fig. 1.1B).

**Figure 1.1 Photographs of sagitally sectioned equine tibia.**

A: Parts of long bone; epiphysis (ep), metaphysis (me) and diaphysis (di). B: The diaphysis consists of cortical (co) bone, and the epiphysis and metaphysis consist of trabecular (tr) bone. Note periosteum (arrow), endosteum (arrowhead) and empty marrow cavity (mc). Photo taken by Yasser Ahmed from a bone belonging to the Anatomy Section, School of Veterinary Science, University of Melbourne.
Histologically, compact bone is formed from organised structures termed osteons or Haversian systems (Fig. 1.2A). Each osteon has a central vascular channel, the Haversian canal, which is surrounded by cylindrical layers of osteocytes arranged in sheets of calcified collagen fibres, lamellae, parallel to the long axis of the bone. Haversian canals are connected to each other and to the medullary cavity through Volkmann’s canals. Unlike compact bone, spongy bone is formed from an interconnecting network of bone trabeculae enclosing bone marrow (Fig. 1.2B).

**Figure 1.2 Histological structure of bone tissue.**
A: Cortical bone; Haversian canals (hc), Volkmann’s canals (vc). B: Spongy bone; bone trabeculae (tr). Note irregular marrow spaces and endosteum. Arrows in A and B indicate osteocytes. Images modified from the University of Texas Medical Branch Cell Biology Graduate Program website (http://cellbio.utmb.edu/microanatomy/bone.htm).

Like all connective tissue, bone tissue consists basically of two components; a cellular component and a matrix component. The cellular component of the bone tissue includes three morphologically and functionally distinct cells; bone-forming cells, osteoblasts, (which mature into a second type of cell, osteocytes) and bone-resorbing cells, osteoclasts. Osteoblasts are cuboidal cells with rounded basal nuclei and are located on bone surfaces (Fig. 1.3A). Osteoblasts originate from undifferentiated mesenchymal cells, osteoprogenitor cells, on the surfaces of the periosteum and endosteum and in bone marrow (reviewed by Aubin et al., 2006). Osteoblasts are highly active cells responsible for deposition of bone matrix, stimulation of matrix calcification
and regulation of osteoclast differentiation, so it is not surprising that these cells have a well developed protein synthesising machinery, rough endoplasmic reticulum (RER) and Golgi apparatus (reviewed by Mackie, 2003). It has been reviewed that after completion of their secretory function, 50-70% of osteoblasts die by apoptosis, while the remaining cells are either trapped into the calcified matrix and become osteocytes or remain on their places as bone-lining cells (Dempester, 2006). The bone-lining cells are thought to act as a barrier to control the fluid flow through the bone matrix, and in response to certain stimulatory factors they become active osteoblasts (Dempester, 2006).

Osteocytes, the mature form of osteoblasts, are embedded inside lacunae (Fig. 1.2) and send several cellular processes through an expansion of the lacunar space, canaliculi, to connect to each other. Osteocytes are less active than osteoblasts and have less developed RER and Golgi apparatus (Eurell and Frappier, 2006). The function of osteocytes is not well-understood, however, they may play a role in calcium homeostasis through remodelling of perilacunar bone (Eurell and Frappier, 2006). Osteocytes are thought to be important for bone maintenance and upon their death, osteoclasts resorb bone containing dead osteocytes (Eurell and Frappier, 2006). Osteoclasts are large multinucleate cells (Fig. 1.3B), which originate from the fusion of mononucleate precursor cells in the bone marrow. Two cytokines, receptor activator for nuclear factor κ B Ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), produced by marrow stromal cells and osteoblasts have been reported as the most important proteins required for osteoclastogenesis (reviewed by Ross, 2006). Old bone is resorbed continually by the enzymatic activity of osteoclasts. A balance between the activity of bone formation (osteoblastic activity) and bone removal (osteoclastic activity) is required for healthy bones. Any imbalance in this relationship is usually associated with pathological conditions such as osteoporosis (reviewed by Eastell, 2006).

The matrix of the bone is formed of two components: an organic part and a mineral part. The organic matrix is protein in nature and contains mostly type I collagen and a lesser amount of non-collagenous proteins. Non-collagenous proteins of the bone matrix can be divided into four categories: proteoglycans such as versican, which are replaced by decorin and biglycan with increased osteogenesis; glycoproteins such as
osteonectin, tetranectin, and tenascin-C and –W; integrin-binding proteins such as osteopontin, fibronectin and thrombospondins; carboxylic acid-containing proteins such as osteocalcin (reviewed by Robey and Boskey, 2006). The mineral matrix consists mainly of hydroxyapatite crystals which includes phosphorus and calcium (reviewed by Robey and Boskey, 2006).

1.1.1.2 Cartilage

Cartilage, which is normally an avascular connective tissue, is present in the body in three major types; hyaline cartilage, fibrocartilage and elastic cartilage. All of these contain a single cell type, the chondrocyte embedded within lacunae in the ECM. Differences between types of cartilage result from differences in their ECM components. Hyaline cartilage is the most common type of cartilage and is present on articular surfaces, in the respiratory tract and in ribs. In locations other than articular cartilage, hyaline cartilage is surrounded by the perichondrium, which is a layer of connective tissue containing blood vessels and nerves. Elastic cartilage has an additional network of elastic fibres and is present in the ear pinnae. Fibrocartilage has no perichondrium

Figure 1.3 Bone cells.

Semi-thin sections from the primary ossification centre (explained in section 1.1.2.2.1) from the head of humerus of an equine foetus showing bone cells. Arrow in A indicates an osteoblast and in B indicates an osteoclast. Bars = 50 μm in (A) and 10 μm in (B). Yasser Ahmed, School of Veterinary Science, University of Melbourne.

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Cartilage, which is normally an avascular connective tissue, is present in the body in three major types; hyaline cartilage, fibrocartilage and elastic cartilage. All of these contain a single cell type, the chondrocyte embedded within lacunae in the ECM. Differences between types of cartilage result from differences in their ECM components. Hyaline cartilage is the most common type of cartilage and is present on articular surfaces, in the respiratory tract and in ribs. In locations other than articular cartilage, hyaline cartilage is surrounded by the perichondrium, which is a layer of connective tissue containing blood vessels and nerves. Elastic cartilage has an additional network of elastic fibres and is present in the ear pinnae. Fibrocartilage has no perichondrium
and has chondrocytes in parallel rows with regular bundles of collagen type I, and is found in intervertebral discs and in the ligaments (Junqueira et al., 1998).

The cartilage ECM is secreted by chondrocytes and contains many components. The components of cartilage ECM are summarised (Bonucci and Motta, 1990) as follows. **Water**: The water content of cartilage is high, comprising about 70% of the cartilage wet weight. **Proteins**: The predominant form of protein in cartilage is collagen, comprising about 30% and 50% of the growth plate and articular cartilage dry weight, respectively (Wardale and Duance, 1994). Collagen type II is the major form of collagen in cartilage tissue, comprising about 95% of the total cartilage collagen, however, cartilage contains smaller amounts of other collagens such as types III, VI, IX, X, XI, XII and XIV (reviewed by Eyre, 2002). **Proteoglycans**: Proteoglycans are composed of glycosaminoglycans, including chondroitin sulphate and keratan sulphate, which are long unbranched chains of disaccharides linked to a protein core that in turn is linked to a long non-sulphated glycosaminoglycan molecule, hyaluronan, by a link protein. The major cartilage proteoglycan is aggrecan, which together with hyaluronan forms large aggregates. The glycosaminoglycans are negatively charged. The polyanionic nature of those aggregates assists in the retention of water within cartilage tissue. **Adhesive proteins**: Adhesive proteins such as fibronectin facilitate adhesion between the chondrocytes and their ECM. The relationships of the major cartilage ECM components to each other are illustrated in Fig. 1.4.

### 1.1.2 Bone Development

Bone development occurs via two different mechanisms, intramembranous and endochondral ossification. Based on these two mechanisms, bones are classified into two types: bones formed by intramembranous ossification, which include flat bones such as the skull, and bones formed by endochondral ossification, which include long bones such as the humerus and femur, short bones such as carpal bones, and irregular bones such as vertebrae.
Figure 1.4 Schematic drawing showing cartilage ECM structure (Eurell and Frappier, 2006).
1.1.2.1 Intramembranous Ossification

Intramembranous ossification is initiated by formation of highly vascular connective tissue sheets rich in undifferentiated mesenchymal cells. These mesenchymal cells differentiate into osteoblasts, which then start to deposit bone matrix (osteoid). Collagen is the first part of osteoid secreted by osteoblasts followed by other components of bone matrix. The osteoblasts become trapped within the newly formed matrix and become osteocytes. The osteoid is calcified forming bone trabeculae, and the structure is called primary spongiosa or woven bone which is immature bone. The primary spongiosa is replaced by lamellar bone (mature bone) either spongy or compact. In areas where spongy bone develops, the spaces between bone trabeculae become bone marrow, however, in areas formed from compact bone, the spaces between trabeculae are filled by bone tissue except for the Haversian canals, then osteons are formed, and the outer surface becomes periosteum (Thompson et al., 1989). These stages are summarised in Fig 1.5.

1.1.2.2 Endochondral Ossification

1.1.2.2.1 Overview

Longitudinal growth of the skeleton results from endochondral ossification. During this process, a cartilage model surrounded by a perichondrium is formed from mesenchymal condensation, which is then replaced by bone and bone marrow (Fig. 1.6). The factors regulating this process are not yet fully understood. Understanding the mechanisms of endochondral ossification will greatly help in understanding many serious disorders in cartilage function.

The process of long bone development in rabbit has been summarised (Rivas and Shapiro, 2002). Two types of centres of ossification can be seen in a developing long bone: a primary ossification centre (POC) appears in the middle of the cartilage model, from where ossification extends towards both ends, and then a secondary ossification centre (SOC) appears in each epiphysis. The cartilage that remains between the POC and SOC is known as the physis or growth plate. Ossification starts when a small
number of chondrocytes in the centre of developing bone undergo hypertrophy, then die.
Through intramembranous ossification, the mesenchymal cells in the perichondrium of
the template except at the two ends differentiate into osteoblasts replacing the
perichondrium by a bony collar, the periosteal bone collar. A periosteal bud, which
contains blood vessels, osteoblasts and bone marrow precursors, invades the centre of
the cartilage model establishing the POC. The osteoblasts deposit bone matrix and
become osteocytes. Similar changes occur in the ends of the developing long bone
without involvement of periosteal bud, establishing the SOC. Primary spongiosa is
formed in the centres of ossification. Primary spongiosa is then replaced by lamellar
bone, either compact or spongy depending on location. Once the POC and SOC have
been established, cartilage contributes to growth in two locations within a long bone.
The cartilage of the physis (physeal growth cartilage; PGC) participates in expansion of
the POC and results in longitudinal bone growth. Underneath the permanent articular
cartilage lies the articular epiphyseal growth cartilage (AEGC), which participates in
expansion of SOC, and results in enlargement and modelling of the epiphysis. After
birth, the cartilage remains only as two structures; PGC and AEGC (Fig. 1.7). Growth
cartilage in both these regions is gradually replaced by bone with maturation of the
skeleton. The AEGC is removed leaving permanent articular cartilage, while the PGC
continues until puberty, when it is completely replaced by bone (Junqueira et al., 1998).

Although normal cartilage is an avascular tissue, the cartilage of the prospective
epiphyses is usually invaded by highly vascular channels, cartilage canals (Fig. 1.8).
Cartilage canals are vascular tubular invaginations from the perichondrium into the
centre of the cartilaginous ends of the developing bone (Rivas and Shapiro, 2002;
Blumer et al., 2004). Cartilage canals provide not only the vasculature and nutrition to
the chondrocytes but also the osteoblast precursors, the undifferentiated mesenchymal
cells that may contribute to formation of the SOC (Burkus et al., 1993; Blumer et al.,
2005; Blumer et al., 2006).
Figure 1.5 Intramembranous ossification.

Section through the maxilla of the foetal pig. Connective tissue sheath (CT), blood vessels (BV), mesenchymal cells (large arrows), osteoblasts (small arrows), osteoid (arrowhead), bone trabecuale (BT). Bar = 75 μm. Photo taken by Yasser Ahmed from a slide belonging to Prof. Eleanor Mackie, School of Veterinary Science, University of Melbourne.
Figure 1.6 Long bone development.
Figure 1.7 Locations of growth cartilage following formation of the secondary ossification centre.

Dr. Thomas Caceci (education.vetmed.vt.edu/.../Labs/Lab8/lab8.htm).
Figure 1.8 Cartilage canal.

Cryosection (A) stained with haematoxylin and eosin and semithin section (B) stained with methylene blue from equine foetal AEGC. Arrows indicate cartilage canals, arrowhead indicates articular surface and HZ indicates the hypertrophic zone. Bar = 125 μm in A and 10 μm in B. Yasser Ahmed, School of Veterinary Science, University of Melbourne.
Two types of cartilage canals have been observed in growth cartilage: “shell” and “communicating” canals. Shell canals are located in the superficial layers of the growth cartilage and communicating canals extend from shell canals and pass downward into the deeper layers (Blumer et al., 2004). As the animal matures, cartilage canals of the growth cartilage chondrify.

1.1.2.2 Growth Cartilage Structure

Growth cartilage is formed of chondrocytes embedded within the ECM, which is produced and controlled by the chondrocytes themselves. Chondrocytes in growth cartilage are present in three zones; reserve, proliferative and hypertrophic zones (Fig. 1.9, Miralles-Flores and Delgado-Baeza, 1990; Jeffcott and Henson, 1998; Adams and Shapiro, 2002; Rivas and Shapiro, 2002). Appearance of growth cartilage varies with the stage of development and between PGC and AEGC, but the same zones are always present. The reserve zone or zone of resting chondrocytes contains rounded to oval chondrocytes containing prominent lipids and densely aggregated glycogen which gradually decreases in the subsequent layers. The zone of proliferative chondrocytes consists of flattened chondrocytes arranged in columns parallel to the long axis of the bone. The hypertrophic zone contains rounded chondrocytes, which become progressively larger with proximity to the ossification front. Although the fate of hypertrophic chondrocytes has been explained by different theories, most of the recent publications have demonstrated that the hypertrophic chondrocytes die by physiological modes before the vascular invasion of the cartilage.

The resting zone has been reported to play an important role in cartilage growth and development; it may contain stem-like cells that give rise to proliferative chondrocytes (Abad et al., 2002; Dowthwaite et al., 2004; Cui et al., 2006) and may secrete factors that inhibit their hypertrophy. Furthermore resting chondrocytes are thought to produce morphogens responsible for the arrangement of the proliferative zone into columns parallel to the long axis of the bone (Abad et al., 2002)
Figure 1.9 Schematic diagram of growth cartilage.
Resting (RE), proliferative (PR), hypertrophic (HY) zones, and zone of vascular invasion (VI). Note calcified ECM (cc).
Modified from http://unu.edu/Unpress/food2/UID06E/uid06e0u.htm.
Chondrocytes in the proliferative zone undergo mitotic division, which decreases with increasing age (Aizawa et al., 1997). Hypertrophic chondrocytes are responsible for secretion of collagen type X (reviewed by Shen, 2005), and they are characterised by expression of high levels of alkaline phosphatase (Vaananen, 1980). The growth cartilage ECM is formed mostly of collagen and proteoglycans, but collagen expression varies from zone to zone. Type II collagen, the predominant structural component of the cartilaginous matrix, is mainly synthesised by chondrocytes in the resting and proliferative zone but its synthesis decreases in the hypertrophic zone. Type X collagen is described as a marker of hypertrophic chondrocytes. Type I collagen is synthesised by cells invading the chondro-osseous border associated with vascular invasion of the cartilage (Yamasaki et al., 2001) as well as by undifferentiated cells of cartilage canals invading the SOC (Blumer et al., 2006).

1.1.2.2.3 Chondrocyte Hypertrophy

In the hypertrophic zone, the cells stop proliferation, increase in volume and become more circular. The physiological enlargement of hypertrophic chondrocytes is associated with an increase in the volume of their organelles especially rough endoplasmic reticulum (RER) and Golgi apparatus, by about 126% with respect to that of the organelles in the early proliferative cells (Buckwalter et al., 1986). Chondrocyte hypertrophy makes an important contribution to bone growth. It has been suggested that chondrocyte hypertrophy is responsible for about 50% of long bone growth and the remaining 50% is due to cellular proliferation and ECM accumulation (Ballock and O'Keefe, 2003). In addition, the difference in growth rate between faster growing bones such as the femur and slower growing bones such as the radius is mostly due to the differences in the increase in size of the hypertrophic chondrocytes (Ballock and O'Keefe, 2003). It has been explained that hypertrophic chondrocytes in human foetal and neonatal cartilage exist as two morphologically different types, light and dark cells (Hwang, 1978). Light and dark chondrocytes have been further characterised in the porcine physis (Wilsman et al., 1981). Although the major component of cytoplasm in
both cells is RER, these cells are distinguished from each other by nuclear morphology and cytoplasmic condensation. The dark chondrocytes had a condensed nucleus, dark cytoplasm and an extensive system of RER as well as many vacuoles. However, the light cells appeared to follow two pathways at the late hypertrophic zone: lamina-enclosed light cells (LEL cells) or stellate cells. The LEL cells were mostly present before capillary penetration and morphologically characterised by a large pale nucleus and pale cytoplasm containing few, widely separated organelles, mostly strands of endoplasmic reticulum. These cells had a smooth ellipsoid shape and were completely filling the lacunae and surrounded by an electron-dense lamina. The stellate cells were not completely filling the lacunae and had many cytoplasmic processes (Wilsman et al., 1981). Carlson et al. (1985) have reported that at the late hypertrophic zone in porcine growth cartilage, the light chondrocytes had three distinct populations, stellate cells, light cells surrounded by an electron-dense rim, which are similar to the previously described LEL cells, and light cells with short processes. More light cells were observed in the AEGC than in the PGC (Carlson et al., 1985). A later electron microscopic study of the late hypertrophic chondrocytes of the porcine growth plate presented similar images for dark and light chondrocytes to those shown previously but the terms “hydrated” and “condensed” cells were used to describe the light and dark chondrocytes, respectively (Farnum and Wilsman, 1987). It was hypothesised that hypertrophic chondrocytes, including terminally differentiated cells, remain viable and active in protein synthesis till vascular invasion of the cartilage (Farnum and Wilsman, 1987). A higher percentage of condensed cells were present in the slower-growing of the two growth plates of the same long bone (Farnum and Wilsman, 1989). From these early morphological studies, it is clear that there are two distinct forms of chondrocytes, light and dark cells. None of these studies has clarified the relationship of light and dark chondrocytes to each other, but Wilsman et al., (1981) suggested that, they may be different stages of differentiation of one cell type. No further mention of light and dark chondrocytes was made in most subsequent publications.
1.1.2.2.4 Regulation of Endochondral Ossification

Biological changes occurring within the growth cartilage are regulated by the interaction between local factors secreted by the chondrocytes and systemic factors, which travel in the blood stream and have receptors in or on the growth cartilage chondrocytes. The local factors include Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), bone morphogenetic proteins (BMPs), Wnt, retinoids, insulin-like growth factor II (IGF-II), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), and transcriptional factors present within the cells such as Sox9 and Runx2. The systemic factors include thyroid hormone, oestrogen, androgens, IGF-I, vitamin D and glucocorticoid (reviewed by van der Eerden et al., 2003). Both PTHrP and Ihh have been reported as the key regulators of chondrocyte proliferation and hypertrophy (Yoshida et al., 2001; Alvarez et al., 2002; Shum and Nuckolls, 2002; Rabie et al., 2003). Indian hedgehog, synthesised by prehypertrophic chondrocytes, stimulates PTHrP expression in the periarticular cartilage and perichondrium, which maintains the proliferative chondrocytes and prevents their hypertrophy (Kobayashi et al., 2002; Shum and Nuckolls, 2002). Independently from the PTHrP pathway, Ihh may stimulate BMP expression, which in turn stimulates chondrocyte proliferation (Minina et al., 2001). Activation of FGF receptor-3 (FGFR3), which is expressed in prehypertrophic chondrocytes, by FGF18, which is expressed by cells in the perichondrium layer, results in prevention of chondrocyte proliferation (Dailey et al., 2003) and hypertrophy (Ornitz and Marie, 2002). Transforming growth factor-β (TGF-β) up-regulates PTHrP (Serra et al., 1999; Pateder et al., 2001; Alvarez et al., 2002). Connective tissue growth factor (CTGF) has been reported as an important stimulator of chondrocyte hypertrophy (Fukunaga et al., 2003). Vascular endothelial growth factor secreted by the late hypertrophic chondrocytes promotes invasion of blood vessels into the cartilage tissue (Gerber et al., 1999). Among transcriptional factors, Sox9 and Runx2 have been reported to play very important roles in the process of endochondral ossification. Sox9 is essential for mesenchymal cell condensation, chondrocytic differentiation, chondrocyte proliferation as well as suppression of prehypertrophic chondrocyte’s conversion into hypertrophic cells (Akiyama et al., 2002),
while Runx2 is essential for inducing chondrocyte differentiation (Stricker et al., 2002) and hypertrophy (Yoshida et al., 2004). It has been reported that PTHrP inhibition of chondrocyte hypertrophy occurs via Sox9 stimulation and Runx2 inhibition (Chung, 2004). Matrix metalloproteinase-13 (MMP-13) is important for normal endochondral ossification as made evident by expansion of the hypertrophic zone in MMP-13 null mice (Inada et al., 2004).

Degradation of ECM is an important step in endochondral ossification. Many factors have been reported as important regulators of cartilage ECM turnover. Metalloproteinase-13 is one of the most important collagen-degrading factors (Wu et al., 2002), but it is not important for aggrecan degradation (Little et al., 2005). A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) is a family of gene products responsible for aggrecan degradation, and it includes ADAMTS-1, -4 and -5 (Nagase and Kashiwagi, 2003). It has been reported that ADAMTS-1 is necessary for aggrecan degradation (Kuno et al., 2000). Cathepsins, intra-cellular lysosomal proteinases, have been reported to play an important role in proteoglycan and collagen degradation (Poole and Mort, 1981). Among different cathepsins, the distribution of cathepsins B and L have spatial and age-related differences in growth cartilage. In equine growth cartilage, animals aged from one month up to two years showed strong staining for cathepsin B in the hypertrophic zone of AEGC. More interestingly, cathepsin L was restricted to the proliferative zone of growth cartilage in most foetuses and neonatal horses as well as in a few growing horses (Glaser et al., 2003). The different pattern of cathepsin distribution in growth cartilage suggests different roles played by different cathepsins in endochondral ossification.

Physiological mineralisation of the growth cartilage ECM is another step in endochondral ossification. It has been reported that hypertrophic chondrocytes produce two types of matrix vesicles similar in appearance and size to each other, and both contain collagen types II and X. However, the matrix vesicles involved in mineralisation strongly express alkaline phosphatase and annexin V, unlike the other ones, which are not involved in the mineralisation process (Kirsch et al., 1997).
1.1.2.2.5 Replacement of Cartilage by Bone

The initiation of bone formation is preceded by breakdown of the transverse septa of matrix between the lowermost lacunae of the hypertrophic chondrocytes, and then capillary sprouts associated with osteogenic cells invade the space vacated by dead chondrocytes (Yamasaki et al., 2001; Eurell and Frappier, 2006). Trabeculae of woven bone are deposited on the calcified cartilage septa at the ends of the growth cartilage (Adams and Shapiro, 2002). The newly formed bone is remodelled by the invading osteoclasts to form the trabecular bone of the metaphysis (Junqueira et al., 1998).

1.1.2.2.6 Development and Maturation of Long Bones in the Horse

Growth cartilage of the horse is histologically similar to other species except that chondrocytes in the AEGC proliferative zone are more randomly distributed rather than forming columns (Jeffcott and Henson, 1998). Furthermore, the cartilage canals gradually diminish after birth and are completely absent in animals aged more than 6 months (Shingleton et al., 1997). Collagen type II is detected in all zones of the foetal and mature AEGC, while collagen type X is found only in the late hypertrophic zone in AEGC in animals aged less than 6 months and in PGC in animals aged less than 12 months (Henson et al., 1996). Alkaline phosphatase activity differs in different locations and decreases with increasing age (Henson et al., 1995). Timing of ossification and epiphyseal closure (when AEGC and PGC disappear except for permanent articular cartilage) of long bones in the horse have been presented (Sisson et al., 1975), and are summarised in Table 1.1.
### Table 1.1 Timing of ossification and epiphyseal closure in the horse

<table>
<thead>
<tr>
<th>Bone</th>
<th>Appearance of POC (day of gestation)</th>
<th>Appearance of SOC (day of gestation)</th>
<th>Epiphyseal closure (determined by macro and/ or micro-examination)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proximal</td>
<td>Distal</td>
</tr>
<tr>
<td>Humerus</td>
<td>60-70 days</td>
<td>290-300 days</td>
<td>290-315 days</td>
</tr>
<tr>
<td>Radius</td>
<td>60-70 days</td>
<td>315-335 days</td>
<td>265-295 days</td>
</tr>
<tr>
<td>Ulna</td>
<td>65-75 days</td>
<td>330 days- after birth</td>
<td>330 days- after birth</td>
</tr>
<tr>
<td>3rd Metacarpal</td>
<td>70-80 days</td>
<td>270-330 days</td>
<td>265-290 days</td>
</tr>
<tr>
<td>Femur</td>
<td>60-70 days</td>
<td>230-300 days</td>
<td>220-245 days</td>
</tr>
<tr>
<td>Tibia</td>
<td>60-70 days</td>
<td>265-300 days</td>
<td>280-300 days</td>
</tr>
<tr>
<td>Fibula</td>
<td>After birth</td>
<td>Soon after birth</td>
<td>325 days- after birth</td>
</tr>
</tbody>
</table>
1.1.3 Osteochondrosis

1.1.3.1 Importance

Osteochondrosis is a developmental orthopaedic pathological condition which arises from disturbances in the normal process of endochondral ossification resulting in retention of cartilage foci within subchondral bone (Fig. 1.10). Osteochondrosis is considered as one of the most common causes of arthritis and other joint diseases of all domestic animals. It causes a high wastage and economic losses to the horse industry all over the world. A better understanding of endochondral ossification is of great importance for understanding the pathogenesis of osteochondrosis (Hernandez-Vidal et al., 1998).

![Figure 1.10 Gross lesion of osteochondrosis.](image)

Photograph of the articular cartilage from the glenoid cavity of the scapula of a 6-month postnatal horse showing a lesion of osteochondrosis (arrow). Khaled Al-Khodair, School of Veterinary Science, University of Melbourne.

1.1.3.2 Aetiology

The aetiology of early osteochondrosis is poorly understood (Jeffcott and Henson, 1998). Multiple factors have been found to play a role in the development of osteochondrosis such as genetics, nutrition, growth rates, endocrinology, biomechanics and toxicosis (Jeffcott, 1991; Muirhead et al., 2003). However, a high energy diet was reported as one of the most common predisposing factors in the development of the disease among domestic animals (Jeffcott and Henson, 1998).
1.1.3.3 Pathogenesis

How the early lesions of osteochondrosis are initiated is still unknown. Jeffcott and Henson (1998) put forward a hypothesis describing the pathogenesis of osteochondrosis. They reported that whatever the primary cause, osteochondrosis is usually associated with abnormalities in either chondrocyte differentiation and/or matrix formation that affect matrix mineralisation and subsequently cartilage resorption. These pathological changes lead to retention of a cartilage core within the bone causing dyschondroplasia, which may not be clinically manifested. Biomechanical factors such as strong exercise or trauma may damage the dyschondroplastic cartilage developing the case into osteochondrosis, which is clinically manifested (Jeffcott and Henson, 1998).

In pigs, it has been suggested that osteochondrosis is a sequela of disturbances in cartilage canal development leading to necrosis of surrounding tissues at the junction of bone and cartilage, providing a weak base for the layers of cartilage above and making it easily damaged (Ytrehus et al., 2004). In the horse, it has been suggested that the existence of cartilage canals in dyschondroplastic lesions from AEGC of postnatal animals aged up to 15 months, despite their absence in normal AEGC from animals aged more than 6 months, may cause increased survival of chondrocytes and failure of their hypertrophy (Shingleton et al., 1997). An important pathological change in osteochondrotic cartilage is the disturbance in cartilage collagen content. For example, a decrease in total collagen, especially collagen type II, and an increase in collagen type VI has been described in osteochondrotic cartilage (Wardale and Duance, 1994).

Although chondrocytes of osteochondrotic animals may appear healthy without necrosis, there is a loss in hypertrophic cells in articular cartilage or cellular disorganisation of the hypertrophic zone in the growth plate, in addition to vascularisation of normally non-vascularised cartilage (Wardale and Duance, 1994). Cathepsin B rich cellular clusters are a common feature of dyschondroplastic cartilage. These clusters are not only associated with abnormal cartilage in the deep zone but are also present in the mid-zone as well as near to the surface of the articular cartilage (Hernandez-Vidal et al., 1998).

Osteochondrotic growth cartilage is associated with alteration in normal regulators of the endochondral ossification process. It has been reported that PTHrP and Ihh
expression increases in equine osteochondrotic articular cartilage, and that may explain the existence of prehypertrophic chondrocytes and failure of their hypertrophy (Semevolos et al., 2002). Expression of aggrecan, collagen types II and X in osteochondrotic articular cartilage remains unchanged (Semevolos et al., 2001).

1.2 Cell Death

1.2.1 Importance of Cell Death

Cell death is a widely distributed programmed phenomenon that occurs in all organs and tissues during foetal and postnatal life as well as in some degenerative diseases. During development, there is a continuous need to remove cells that are transiently useful to the body. In adult tissues, a balance between cell proliferation and cell death is required for regulating the total number of cells. Hence, elimination of cells by their death has no effect on the function of whole tissues. Moreover, cell death is an important vehicle of physiological regulatory mechanisms involved in different organs and tissues, for example in the involution of the mammary gland (Tatarczuch et al., 1997). In addition, cell death is responsible for removal of unwanted or dangerous cells such as cancer cells, varieties of infected cells or immune cells that recognise infected or damaged cells. Although cell death is a useful process for normal homeostasis, too much or too little of it causes pathological disorders.

1.2.2 Historical Overview of Cell Death

Cell death is an important event in normal tissue development as well as in many diseases affecting different tissues. Early works (Schweichel and Merker, 1973) classified cell death into two categories. The first is physiological necrosis, which occurs during embryogenesis, and the second is induced necrosis that results from toxic agents. The physiological necrosis was sub-classified into three types. Type 1 is found mostly in isolated cells and characterised by nuclear condensation, cellular fragmentation, and phagocytosis of the resulting fragments by their neighbouring cells. Type 2 occurs primarily in tissues which are removed “in toto” and is characterised by formation of lysosomes and abundant autophagic vacuoles within the dying cells,
followed by cellular destruction and phagocytosis. Type 3 is characterised by swelling of membranous organelles such as mitochondria and the formation of empty spaces within the cytoplasm that fuse with each other and connect with extracellular spaces. These developing cellular changes usually end with cellular fragmentation into smaller pieces, cell debris which disappears later without the involvement of the lysosomai system or phagocytosis. The main example given for this type of cell death was the hypertrophic chondrocytes in growth cartilage (Schweichel and Merker, 1973).

The current understanding of different forms of cell death includes different terminology. Apoptosis marks features previously described as type 1 physiological necrosis, type 2 physiological necrosis is referred to as autophagic cell death, however, type 3 is still to be characterised. Induced necrosis is currently known as necrosis. Except for apoptosis, the biochemical pathways leading to different the forms of cell death have not been well studied. Understanding the biochemical pathways of these non-apoptotic forms of cell death is of a great importance not only for understanding the theory of development, but also for understanding many degenerative diseases and subsequently improving therapeutic approaches for these diseases.

1.2.3 Apoptosis

1.2.3.1 Characteristics of Apoptosis

Apoptosis is a form of programmed cell death. The definitive identification of apoptosis is morphological at the ultrastructural level (Kerr et al., 1972). Apoptosis is morphologically marked by common unmistakable features (Fig. 1.11) such as shrinkage in cell volume, regular condensation of the nucleus followed by breaking of the nucleus into pieces (Wyllie and Duvall, 1992). The cytoplasm undergoes condensation and fragmentation into membrane-bound apoptotic bodies containing multiple numbers of nucleosomes, the DNA unit structure, which are rapidly phagocytosed by specialised phagocytic or adjacent cells (Kataoka and Tsuruo, 1996). These changes are extremely rapid and take from a few minutes to a few hours (Fraser and Evan, 1996).
Apoptosis is described as occurring in two main phases. The first phase is the effector phase, in which intracellular proteases such as caspases are activated and mitochondrial dysregulation occurs. The second phase is the degradation phase, in which the nucleic acids and proteins undergo extensive breakdown (Aigner, 2002).

1.2.3.2 Regulation of Apoptosis

A roundworm, Caenorhabditis elegans (C. elegans), was the first target of intensive genetic studies aimed at understanding the mechanism of apoptosis. The mature worm consists of about 1000 cells. During development, 131 cells die by apoptosis. Mutations in two genes, Ced-3 and Ced-4, can inhibit cell death in these worms, however, mutations in Ced-9 cause all cells to die (reviewed by Fraser and Evan, 1996). A protease, homologous to the Ced-3 gene product, interleukin-1 converting enzyme (ICE), was first identified in mammals (Yuan et al., 1993), then used to induce apoptosis in cultured fibroblasts (Miura et al., 1993). A series of proteases, caspases, which are known to be required for apoptosis were then discovered; about 14 members of the caspase family have been identified in mammals.

Caspases are intracellular proteases present as inactive precursors. Activation of an initial caspase leads to cascade activation resulting in apoptosis. Caspases are divided into three groups. Group 1 caspases (caspase-1, -4, -5, -11, -12, -13 and –14) have no significant role in apoptosis but function by activation of pro-inflammatory cytokines. Group 2 caspases (caspase-2, -8, -9, and –10) are important for apoptosis, which function by activation of endonucleases resulting in chromatin breakdown. Group 3 caspases (caspase-3, -6, and –7) are known as effector caspases and play a key role in enzymatic cleavage of many cellular proteins (reviewed by Mirkes, 2002). Caspases, the main apoptosis regulators, have two pathways to be activated: the mitochondrial (intrinsic) pathway that occurs via caspase-9 activation, and the death receptor (extrinsic) pathway which occurs via caspase-8 and caspase-10 activation. Both pathways join at the level of caspase-3 cascade activation (reviewed by Bursch, 2004).
Figure 1.11 Morphological characteristics of cells undergoing apoptosis.

Electron micrograph of isolated lymph. Note formation of many apoptotic bodies (1-8) and geometric chromatin condensation (arrowheads). Bar = 2 µm. L. Tatarczuch, School of Veterinary Science, University of Melbourne.
In healthy mammalian cells, the outer membranes of mitochondria express Bel-2 (functionally similar to Ced-9 in C. elegans). Bcl-2 binds to a cytoplasmic protein, apoptotic protease-activating factor-1 (Apaf-1), and prevents its interaction with cytochrome C. In the intrinsic pathway, damage of the mitochondria releases Apaf-1 from Bcl-2 leading to released cytochrome C and Apaf-1 binding to caspase-9, forming a complex termed an apoptosome. This structural complex stimulates a caspase cascade resulting in apoptosis. However, in the extrinsic pathway, activation of specific membrane receptors, CD95/FAS leads to activation of caspase-8 and -10 which in turn activate other caspases resulting in apoptosis (reviewed by Mirkes, 2002).

Although activation of caspases is necessary for apoptosis to occur, it seems that these proteases cannot be considered as an indicator for apoptosis. In addition to their clear role in apoptosis, caspases have recently been reported to regulate a variety of other functions such as cell survival, cell movement, receptor internalization, cell proliferation and differentiation (Algeciras-Schimnich et al., 2002). Thus cells that have a high caspase activity are not necessarily dying cells. Moreover, inhibition of caspases using broad-spectrum anti-caspases results in inhibition of apoptosis but not cell death and may induce necrosis-like cell death characterised by cytoplasmic vacuolation and minimal nuclear changes (Kitanaka and Kuchino, 1999).

1.2.4 Autophagic Cell Death

Autophagy is a physiological process, which occurs routinely in most cells for degradation of expired and unwanted proteins or organelles. However, extensive degradation of specific proteins or organelles essential for cell survival results in autophagic cell death. Autophagic cell death is a form of programmed cell death that is morphologically distinct from apoptosis and occurs during development (Hwang et al., 2004; Yu et al., 2004). In cells that undergo autophagic cell death, there is formation of many vacuoles within the cytoplasm and cytoplasmic contents show self-digestion due to lysosomal activity (Bursch et al., 2000). The role of caspases in autophagic cell death is a point of controversy. Some studies have shown that caspases may be required for autophagic cell death (Martin and Baehrecke, 2004).
Others have reported that autophagic cell death is a caspase-independent form of programmed cell death (Kitanaka and Kuchino, 1999; Lockshin and Zakeri, 2002). Moreover, this form of cell death has been described as being inhibited by caspases (Hwang et al., 2004; Yu et al., 2004).

1.2.5 Necrosis

In contrast to apoptosis, which is an active form of programmed cell death, necrosis is a passive non-programmed cell death. Cells undergoing necrosis have different morphological changes: swelling of the cytoplasm and organelles such as mitochondria (due to disturbances in the ability of their plasma membranes to control passage of ions and water) and leaking of the cellular contents into the surrounding ECM, causing inflammatory reactions (Wyllie and Duvall, 1992; Majno and Joris, 1995). Interestingly, a form of cell death sharing some features of apoptosis (chromatin condensation and nuclear fragmentation) and necrosis (disturbance in the cell membrane and cytoplasmic swelling), has been described in cultured fibroblasts and termed "aponecrosis" (Formigli et al., 2000). Aponecrosis may represent apoptotic cells undergoing necrosis due to the lack of phagocytic activity in culture.

A summary of morphological differences between different forms of cell death is presented in table 1.2.
Table 1.2 Summary of morphological characteristics of apoptosis, necrosis and autophagic cell death.

<table>
<thead>
<tr>
<th>Features</th>
<th>Apoptosis</th>
<th>Autophagic cell death</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nuclear chromatin</strong></td>
<td>Chromatin condensed into sharply circumscribed round or crescent masses at the margin of nuclear membrane</td>
<td>Chromatin slightly condensed or extensively compacted into single pyknotic mass in the centre of nucleus</td>
<td>Moderately condensed in irregular aggregates</td>
</tr>
<tr>
<td><strong>Nuclear membrane</strong></td>
<td>Progressively convoluted; initially well preserved, later discontinuous</td>
<td>Well preserved</td>
<td>Ruptured</td>
</tr>
<tr>
<td><strong>Cytoplasm</strong></td>
<td>Condensed; well preserved organelles compacted together (RER may be focally distended)</td>
<td>Contains numerous autophagocytic vacuoles</td>
<td>Cytoplasm and organelles grossly swelled and vacuolated</td>
</tr>
<tr>
<td><strong>Cell membrane</strong></td>
<td>Well preserved; often convoluted</td>
<td>Well preserved</td>
<td>Ruptured early</td>
</tr>
<tr>
<td><strong>Final stage</strong></td>
<td>Nucleus and cytoplasm fragmented into membrane bound apoptotic bodies</td>
<td>Organelles degraded (amorphous cytoplasm); collapsed nucleus</td>
<td>Cytoplasm and organelles disintegrate; chromatin dissolved (nuclear ghost)</td>
</tr>
<tr>
<td><strong>Timing of nuclear and cytoplasmic changes</strong></td>
<td>Nuclear changes precede cytoplasmic alterations</td>
<td>Cytoplasmic changes precede nuclear alteration</td>
<td>Cytoplasmic changes precede nuclear alterations</td>
</tr>
<tr>
<td><strong>Removal of cellular remnants</strong></td>
<td>Early phagocytosis</td>
<td>Late phagocytosis</td>
<td>Late phagocytosis</td>
</tr>
</tbody>
</table>

(Wyllie *et al.*, 1980; Bursch, 2001)
1.2.6 Physiological Cell Death in Growth Cartilage

It is generally accepted that terminally differentiated hypertrophic chondrocytes have to be eliminated before the vascular invasion of cartilage ECM. However, the mechanism of chondrocyte removal is still a point of mystery and controversy.

From the early ultrastructural studies it became clear that there are two distinct forms of chondrocytes, dark and light cells (Hwang, 1978; Wilsman et al., 1981; Carlson et al., 1985; Farnum and Wilsman, 1987). However these studies did not clarify how these cells die. Wilsman et al (1981) described disintegrating light and dark cells. They were not described as apoptotic, nor did their appearance fit with the morphology of apoptotic cells. In the subsequent studies many authors reported apoptosis as the fate of hypertrophic chondrocytes in growth cartilage during normal endochondral bone formation (Gibson et al., 1995; Hatori et al., 1995; Ohyama et al., 1997; Adams and Horton, 1998; Gibson, 1998; Ploumis et al., 2004). These studies depended mostly on detection of DNA strand breaks using different markers such as the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) and DNA gel electrophoresis, and very few studies have examined the morphology of dying chondrocytes. TUNEL-positive cells were detected in the hypertrophic zone in mice (Adams and Horton, 1998), rat (Ploumis et al., 2004) and chicken (Hatori et al., 1995) growth cartilage. In addition, some studies showed that also some cells in the proliferative zone stained positively for TUNEL (Hatori et al., 1995; Ploumis et al., 2004). However, some studies reported that only the degenerating cells, close to the cartilage-bone interface are TUNEL-positive (Zenmyo et al., 1996). Although a few studies have described the morphological appearance of chondrocytes as apoptosis (Zenmyo et al., 1996; Ploumis et al., 2004), the examples they have shown do not match the morphological characters of apoptosis described by Kerr et al., (1972).

Roach and colleagues have presented the results of a series of very interesting ultrastructural studies. In these studies, they have described alternative pathways to apoptotic elimination of hypertrophic chondrocytes in growth cartilage. In 2000, Roach and Clarke reported that cell death in rabbit growth plate chondrocytes is not confined to apoptosis, and they described two alternative forms of physiological cell
death (PCD): "dark" and "paralysed" cells (Roach and Clarke, 2000). Both cell types have been reported to have a nuclear condensation pattern different from apoptosis. The nucleus of paralysed or dark cells had a patchy nuclear condensation. The main feature of dark cells was continuous removal of cytoplasm “blebbing”, while the main feature for paralysed cells was disappearance of cytoplasmic organelles except for worm-like inclusions of RER and Golgi apparatus. Four years later, Roach and colleagues re-defined “dark” chondrocytes, introducing the term “chondroptosis” (Roach et al., 2004). More recently Shapiro and associates have hypothesised that hypertrophic chondrocytes die by autophagic cell death (Shapiro et al., 2005).

1.3 Chondrocyte Culture
1.3.1 Three Dimensional Chondrocyte Culture

Growth cartilage is an organised tissue consisting of chondrocytes and ECM. The chondrocytes undergo a series of orchestrated events starting with proliferation and ending with hypertrophy. The terminally differentiated chondrocytes die and the cartilage ECM is replaced by bone and bone cells. These changes are usually controlled by the interaction between systemic hormones and local growth factors. The identification of the precise mechanism of action of these factors on chondrocyte PCD is not completely understood due to the absence of an established in vitro model in which the chondrocytes show the same form of cell death seen in vivo (Roach et al., 2004).

It is well known that chondrocytes cultured in a monolayer rapidly divide, lose their characteristic spherical phenotype and become fibroblast-like cells. This change in the chondrocytic morphology is usually associated with a shift from the expression of the cartilage specific collagen type II in favour of the expression of collagen type I, a collagen typical of chondrogenic precursor cells (Stewart et al., 2000; Malpeli et al., 2004). This phenotypic change in chondrocytes is frequently described as in vitro dedifferentiation. Hence, several in vitro models have been developed to maintain the differentiated state of chondrocytes in a cartilage-like tissue mimicking the regulated biological changes that occur in growth cartilage. Such models facilitate the study of the different roles of the growth factors and hormones in chondrocyte growth and maturation. These systems involve 3-
dimensional (3-D) culture, and include culturing of chondrocytes in scaffolding materials, such as agarose (Bruckner et al., 1989; Bohme et al., 1992; Quarto et al., 1992; Hunter et al., 1993; Robson et al., 2000), collagen (Yasui et al., 1982; Chaipinyo et al., 2002) or alginate (Yaeger et al., 1997; Masuda et al., 2003). In addition, pellet culture as a method of maintaining chondrocyte phenotype was originally developed in the late 1980s by Kato et al. and has since been modified and widely used by many researchers.

1.3.1.1 Chondrocyte Agarose Suspension Culture

In the agarose suspension culture system, a suspension of cells in medium is mixed with agarose, plated in agarose pre-coated petri dishes, allowed to solidify at 4°C and transferred to a 37°C incubator where the cells grow under 5% carbon dioxide (CO₂) conditions. Unlike culturing in monolayer cultures, the attractive feature of culturing chondrocytes in agarose is that this method permits the cells to exist in a round configuration which is known to favour the maintenance of the cartilage phenotype and promote in vitro chondrogenesis (Hunter et al., 1993). Furthermore, cells that had dedifferentiated in monolayer cultures have been reported to re-differentiate, secreting collagen type II, when cultured in agarose in a serum-free medium supplemented with osteogenin and a mixture of growth factors: insulin, FGF-2, platelet-derivatived growth factor (PDGF) and epidermal growth factor (Harrison et al., 1991). Chondrocytes cultured in an agarose suspension system in serum-containing medium eventually become morphologically hypertrophic and express the two markers of hypertrophic chondrocytes, collagen type X and alkaline phosphatase (Bruckner et al., 1989; Hunter et al., 1993). Similarly, serum-free medium supplemented with thyroxine (Bohme et al., 1992), osteogenic protein-1 (Chen et al., 1995) or triiodothyronine (T3,Quarto et al., 1992) supports chondrocyte hypertrophy. Although chondrocytes cultured at high-density in a completely serum-free medium remain viable and express many of the biochemical and morphological characteristics of resting cells, they fail to undergo hypertrophy (Bruckner et al., 1989). Many studies have described TGF-β as a potent stimulator of in vitro chondrogenesis. Unexpectedly, some investigators have found that TGF-β in serum-free agarose culture not only fails to stimulate proliferation or matrix production but also causes the cells to dedifferentiate in a manner similar to that seen in monolayer
cultures; the presence of serum prevents this phenomenon (Tschan et al., 1993). There are few reports on the nature of the ECM synthesised by chondrocytes cultured in agarose gels. It has been reported that while hydrocortisone stimulates aggrecan synthesis, it has a negative effect on the low molecular weight proteoglycans (Almqvist et al., 2000). Addition of β-glycerophosphate (Hunter et al., 1993) or adenosine tri-phosphate (ATP, Elfervig et al., 2001) to the culture induces calcification in the ECM.

1.3.1.2 Chondrocyte Collagen Gel Culture

Few investigators have used this method of chondrocyte culture. In the collagen gel culture system, the chondrocyte suspension is mixed with a collagen-I gel, placed in culture plates, and allowed to grow in a humidified incubator. Chondrocytes cultured in this way maintain their cartilage phenotype (Yasui et al., 1982). Chondrocytes cultured in collagen system proliferate in a similar manner when grown in either serum-containing medium or serum-free medium supplemented with a mixture of growth factors: TGF-β1, IGF-1 and FGF-2 (Chaipinyo et al., 2002). Chondrocytes grown for two weeks in monolayer can re-differentiate after one week in collagen gel culture (Kuriwaka et al., 2003).

1.3.1.3 Chondrocyte Alginate Bead Culture

In this system of culture, the chondrocytes are encapsulated in alginate beads. This method includes several steps. The isolated chondrocytes are mixed with a solution of alginic acid to form a homogeneous suspension. The suspension is drawn into a syringe and pushed slowly through a needle attached to the syringe. The drops are allowed to polymerise in a solution of calcium chloride. The beads are finally washed thoroughly in a buffer solution before rinsing and culturing in serum-containing medium (De Ceuninck et al., 2004). To understand the mechanism of in vitro chondrogenesis, extensive studies on chondrocyte behaviour and ECM components have been undertaken using alginate beads. It has been shown that the chondrocytes cultured in alginate beads can proliferate and synthesise abundant amounts of ECM without addition of any exogenous factors (Almqvist et al., 2000;
Furthermore, addition of ascorbic acid, which is commonly used in chondrocyte culture, not only stimulates chondrocyte proliferation and maintains the chondrogenic characteristics of the cells (Kim et al., 2003), but also increases ECM synthesis (Awad et al., 2003). It is well known that changes in O₂ tension have a strong influence on the metabolism and survival of articular chondrocytes. Viability of equine chondrocytes cultured for 14 days in alginate beads is greatly affected by changes in O₂ tension, increasing with low (5%) O₂ and decreasing with high (21%) O₂ tension (Schneider et al., 2004).

The advantage of this culture system is that alginate beads allow cell proliferation, providing a useful alternative tool to monolayer culture (Graff et al., 2003; Lee et al., 2003). Human chondrocytes passaged twice in monolayer culture have been shown to re-express the ECM markers, collagen type II and aggregan when cultured for two weeks in alginate beads (Lemare et al., 1998). Foetal cells are preferred for alginate bead culture over adult cells as proteoglycan synthesis decreases with advancing age (Kamada et al., 2002).

1.3.1.4 Chondrocyte Pellet Culture

Although collagen, agarose and alginate beads maintain a chondrocytic phenotype and support the terminal differentiation of chondrocytes into hypertrophic cells, they have some disadvantages. For example, the number of cells encapsulated in each alginate bead is difficult to control, increasing the variability between experiments. In addition, the staining of ECM with toluidine blue is similar to that of the alginate matrix making it difficult to distinguish between chondrocyte ECM and alginate (Yung Lee et al., 2001). The non-pellet 3-D cultures are time-consuming methods, as they require many steps. Furthermore, the use of many chemicals in the alginate culture system is not only expensive but also may change the pH of the surrounding environment affecting cell growth and metabolism.

Among the different culture techniques, the pellet culture system offers many advantages over the others. Culturing of chondrocytes in 3-D pellets is the simplest method for obtaining a cartilage-like tissue in vitro. In this system, the isolated chondrocytes are simply centrifuged in 15-ml centrifuge tubes to form pellets and the pellets are allowed to grow in a humidified incubator at 5% CO₂ (Kato et al., 1988;
Kato and Iwamoto, 1990). Pellet cultures have the advantage of being easy to prepare and maintain in a simple culture environment without a need for specially designed bioreactors. In addition, this culture system allows cell-cell interaction mirroring pre-cartilage condensation during foetal development of growth cartilage. Interestingly, chondrocytes cultured as pellets are usually able to respond strongly to different growth factors and cytokines, which are known to affect chondrocyte metabolism. Moreover, the marker of hypertrophic chondrocytes, collagen type X, is more rapidly expressed in the pellet culture system than in other systems (Yang et al., 2004). Therefore, chondrocyte pellet culture is an extremely useful tool for studying chondrocyte hypertrophy in vitro.

Chondrocytes isolated from many different species have been used as a source for pellet cultures; the species include mouse (Okubo and Reddi, 2003), rat (Ballock et al., 1993; Ballock and Reddi, 1994), rabbit (Kato et al., 1988; Iwamoto, 1989; Kato and Iwamoto, 1990), pig (Graff et al., 2003), ox (Xu et al., 1996; Croucher et al., 2000), and more recently, human (Jakob et al., 2001; Yung Lee et al., 2001; Malda et al., 2003; Malda et al., 2004; Malpeli et al., 2004). At the time of initiation of the work for this thesis there were no publications describing pellet culture for equine chondrocytes.

The 3-D pellet culture has been used for different purposes. Many investigators have used 3-D pellet cultures to study the effects of different growth factors and hormones on the process of chondrogenesis generally and chondrocyte hypertrophy especially. However, no study to date has examined whether chondrocyte non-apoptotic PCD occurs in pellet culture, let alone examining the effects of growth factors and hormones on this process.

It has been shown that the addition of FGF-2 to chondrocyte pellet culture at the transition stage between matrix maturation and cell hypertrophy significantly diminishes the increases in both alkaline phosphatase and calcium content in the pellets (Iwamoto, 1989; Kato and Iwamoto, 1990). The addition of thyroxine has been reported to increase both markers of chondrocyte hypertrophy, collagen type X and alkaline phosphatase (Ballock and Reddi, 1994), induce the arrangement of chondrocytes into columns and down-regulate Sox9 mRNA expression (Okubo and Reddi, 2003). Studies examining the effect of TGF-β on chondrogenesis have
reported varying results. TGF-β1 alone has been reported to decrease the pellet size, while ultrasound had little effect on the pellet size but exposure of the pellets to both at the same time has been found to stimulate cell proliferation and increase the DNA content after 10 days of culture (Ebisawa et al., 2004). Furthermore, the terminal differentiation of the chondrocytes into hypertrophic cells is inhibited by addition of exogenous TGF-β1 to the pellet cultures (Ballock et al., 1993). One study showed that TGF-β has no effect on collagen type II deposition in chondrocyte pellet cultures (Xu et al., 1996) while in another study it was shown that TGF-β1 increases collagen type II production (Jakob et al., 2001). Interestingly, it has been reported that a single dose of ATP or uridine triphosphate added to serum-containing medium on the first day of culture is sufficient to increase both collagen and proteoglycan deposition in pellet cultures over several weeks (Croucher et al., 2000).

1.3.2 Cell Death in Cultured Chondrocytes

Apoptosis is the only form of PCD that has been described in cultured chondrocytes up till now. Chondrocytes are highly social cells; their survival in culture depends mostly on survival factors secreted by neighbouring chondrocytes. Chondrocyte are programmed to kill themselves once these survival factors become unavailable or insufficient (Ishizaki et al., 1994). It has been shown that apoptosis (as confirmed by electron microscopy and TUNEL) is greater in chondrocytes cultured in gels at low density in comparison to those cultured at high density under the same conditions. However, treatment of low-density cultures with a conditioned medium from high-density cultures results in decreasing the apoptotic cell population (Ishizaki et al., 1994). The pathway of apoptosis in chondrocytes has not been clearly addressed. To study the mechanism of apoptosis in chondrocytes, investigators have induced or inhibited cell death by apoptosis in monolayer culture. Several factors and conditions have been reported to induce apoptosis in chondrocytes cultured in monolayer, for example, low serum concentration (Gruber et al., 2000), collagenase (Lo and Kim, 2004), hydrogen peroxide (Lo and Kim, 2004), inorganic phosphate (Mansfield et al., 2003), nitric oxide (Blanco et al., 1995) anti-Fas (Hashimoto et al., 1998; Kuhn et al., 2003). Tumour necrosis factor alpha has been reported to induce apoptosis in cultured chondrocytes (Cho et al., 2003),
although it has been reported in another study to prevent nitric oxide-induced apoptosis in cultured chondrocytes (Relic et al., 2002), aza-cytidine (Cheung et al., 2003), and corticosteroids (Nakazawa et al., 2002). Interleukin-1β (IL-1β) also induces apoptosis (Kuhn et al., 2003) and has been reported to antagonise IGF-1’s anti-apoptotic effect (Kataoka and Tsuruo, 1996), although, IL-1β has been reported to prevent CD95-dependent apoptosis in another study (Kuhn et al., 2000). Furthermore, this anti-apoptotic effect of IL-1β is dependent on neither nitric oxide nor prostaglandins (Kuhn et al., 2000). Staurosporine, a protein kinase inhibitor, has also been reported as a potent apoptosis inducer in cultured chondrocytes (Teixeira et al., 2001; D’Lima et al., 2004).

A wide variety of chondrocyte apoptosis inhibitors have been identified. It has been reported that, in addition to its anabolic effect on chondrocytes, IGF-1 inhibits nitric oxide-induced apoptosis in cultured chondrocytes (Oh and Chun, 2003). Furthermore IGF-1 and PDGF have an anti-apoptotic effect on human intervertebral disc cells (Gruber et al., 2000).

Although apoptosis can be assessed by a variety of methods, electron microscopy is still the superior tool to differentiate between apoptosis and other non-apoptotic forms of cell death. However, only few studies have used electron microscopy to describe apoptosis in chondrocytes (Ishizaki et al., 1994). TUNEL staining is a widely used method for apoptosis identification (Garimella et al., 2004; Zermeno et al., 2006) although, there are some doubts about the ability of this method to differentiate between apoptosis and other forms of cell death such as necrosis (Charriaut-Marlangue and Ben-Ari, 1995). Annexin V-FITC binding, measured by flow cytometry has been used by some authors to detect early stages of apoptosis in cultured chondrocytes (Takahashi et al., 2003). DNA fragmentation analysis in agarose gel is another method to detect chondrocyte apoptosis (Kolettas et al., 2001; Mansfield et al., 2003).
1.4 Aims of the study

Terminal differentiation of chondrocytes into hypertrophic cells is an obligatory step in the pathway of endochondral ossification. It is well known that the terminally differentiated chondrocytes die before vascular invasion of the cartilage matrix and replacement of the cartilage by bone. However, the mechanism of chondrocyte death is not yet understood. Early studies presented two types of hypertrophic chondrocytes but they did not explain how these cells die. More recently studies have ignored this morphological diversity of chondrocytes and claimed that hypertrophic chondrocytes die by apoptosis, on the basis of studies using non-morphological methods depending on detection of DNA strands breaks. Recently, a published ultrastructural study has confirmed that the rabbit hypertrophic chondrocytes undergo non-apoptotic forms of cell death (Roach and Clarke, 2000). However, no studies have yet explained how light and dark cells relate to each other, nor presented a clear morphological description of the process of PCD undergone by hypertrophic chondrocytes. Moreover as pointed out by Roach et al., (2004) studying these different forms of cell death is difficult due to the lack of an in vitro model in which PCD of hypertrophic chondrocytes can be replicated.

The aim of the current study was to investigate the mechanisms of physiological death in equine hypertrophic chondrocytes as an initial step to understanding the mechanism of osteochondrosis in horses. This includes two broad strategies. The first strategy is to identify morphologically the process of PCD of chondrocytes in equine growth cartilage during foetal and postnatal development in vivo. The second strategy is to develop a system of tissue culture in which chondrocytes isolated from equine growth cartilage can be induced to undergo hypertrophy and the same mode of PCD seen in growth cartilage in vivo. This culture system will be used to characterise different forms of chondrocyte death in vitro.
CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Animals

Growth cartilage was obtained from horses throughout development and growth. Nine foetuses of pregnant mares killed at the Melbourne local abattoir were collected and the stages of gestation (3, 6, 8, 9, 10 months) were estimated on the basis of crown/rump length (Fig. 2.1). Samples were also obtained from one neonatal foal (7 days), three growing horses (6, 12 months) and one adult (17 years) horse, which had died from diseases not related to the skeletal system at the Veterinary Clinic and Hospital, University of Melbourne. Table 2.1 shows the investigations undertaken on different samples presented in this study.

2.1.2 Consumable Items, Equipment and Software

Chemicals and other reagents were obtained from Sigma-Aldrich (St Louis, Mo, USA) unless otherwise specified in Appendix 2.1 at the end of this chapter. The sources of kits are listed in Appendix 2.2. Sources of tissue culture plasticware, specialised equipment and software are listed in Appendix 2.3.

2.2 Methods

2.2.1 Specimen Collection for Light and Electron Microscopy

Specimens used for this study were from the growth cartilage of the head of the humerus at different stages of development. The specimens collected from foetal foals (3, 6, 8, 9, 10 months) extended from the articular surface to the POC. Two specimens were collected from the 6-month postnatal foal; one extended from the articular surface to the SOC, and the other from the SOC to the POC. The stages of development of the bones and the locations from which specimens were taken are illustrated schematically in Fig. 2.2.

Skin, muscles and tendons were removed and joints were opened carefully without touching the cartilage.
Figure 2.1 Estimation of foetal age (Noden and de Lahunta, 1985).
Table 2.1 Investigations undertaken on each specimen.

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<thead>
<tr>
<th>Investigation</th>
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N.B: m- refers to month, neonat- refers to neonatal, and y- refers to years.
Figure 2.2 Stages of development in the growth cartilage of the head of the humerus corresponding to the animals collected for the present project.

A: Early foetal stage (3, 6 months post-conception). B: Late foetal stage (8, 9, 10 months post-conception). C: Postnatal stage (6 months). Rectangles indicate the location from which specimens were collected for histology at each stage of development. CE – chondroepiphysis, POC – primary ossification centre, SOC – secondary ossification centre, AEGC – articular epiphyseal growth cartilage, PGC – physeal growth cartilage.
Whole cartilage with some adjacent subchondral bone was excised and rapidly fixed in 5% glutaraldehyde/4% paraformaldehyde (pH 7.4) in 0.2 M cacodylate buffer (0.2 M sodium cacodylic acid; pH 7.4) for three days at 4°C. Specimens were demineralised in 0.33 M ethylenediaminetetraacetic acid (EDTA, pH 7.4) for about three weeks.

2.2.2 Specimen Processing for Light and Electron Microscopy

Demineralised samples were washed thoroughly in 0.1 M cacodylate buffer (pH 7.4), post-fixed in 1% osmium tetroxide/1.5% potassium-ferrocyanide for 2-3 hours in a rotator at room temperature and washed in 0.1 M cacodylate buffer 3 times (30 minutes each). Specimens were serially dehydrated through ascending grades of acetone; 70% acetone (1 hour at least), two changes of 95% acetone (30 minutes each) and four changes of 100% acetone (30 minutes each). After being infiltrated with a mixture of normal Spurr’s resin and 100% acetone 1:1 (overnight), 2:1 (during the day) and two changes of pure resin (12 hours each), samples were embedded in pure Spurr’s resin and allowed to polymerise for 48 hours at 60°C. Semi-thin sections (0.5 µm) were cut with a Reichert OmU2 ultramicrotome. Sections were stained with 1% methylene blue in 1% borax, heated for 5-10 seconds at 100-150°C on a hot plate, washed with distilled water, dried on a filter paper, mounted with distyrene dibutylphthalate and xylene (DPX) and viewed with a light microscope.

Ultra-thin sections were cut, stained with 5% aqueous solution of uranyl acetate for 10 minutes, washed with distilled water and stained with Reynold's lead citrate for 10 minutes. Sections were examined under a transmission electron microscope (TEM; Philips 300). All the electron microscopy steps including ultra-thin sectioning of embedded specimens, examining and photographing of sections were carried out by Ms Liliana Tatarczuch, School of Veterinary Science, University of Melbourne.

2.2.3 Counts of Different Cell Types in Equine Growth Cartilage

Counts of four categories of chondrocytes (as described in section 3.2.2) were conducted on semi-thin sections taken from the PGC and AEGC. Counts were
undertaken using a 100x oil immersion lens in a light microscope. The counts were taken from two regions of the growth cartilage; one region was defined as the mid-point, and was half-way between the last few flattened proliferative cells and the ossification front, and the second region was the last three lacunae before the ossification front (Fig. 2.3). For the mid-point, all cells in the section lying on the line designated as the mid-point were counted. All cells lying within the last three lacunae were counted. For each region, all cells were allocated into one of the four categories (section 3.2.2) and expressed as a percentage of the total cell number.

Figure 2.3 Locations of cell counts undertaken from equine growth cartilage. Lines indicate: (1) last proliferative chondrocytes, (2) ossification front, (3) half-way between (1) and (2), i.e. the mid-point; the last three lacunae are located between (4) and (2). Counts were taken at two locations: the mid-point and the last three lacunae.
2.2.4 Chondrocyte Isolation and Culture

2.2.4.1 Chondrocyte Isolation

Metacarpophalangeal and metatarsophalangeal (fetlock) joints from the left and right fore and hind limbs of foetal, neonatal, growing and adult horses were opened under aseptic conditions in a biological safety cabinet, and the cartilage from the epiphyses excised. In some specimens the SOC was present but in others it was not. In each case, the excised cartilage included the articular surface, and excluded any bone tissue and the zone of hypertrophy; it contained primarily resting, pre-proliferative chondrocytes. The cartilage was sprayed with 70% ethanol and dissected into small pieces. Cartilage from all joints from each animal was pooled. Cartilage fragments were thoroughly washed in serum-free Dulbecco's Modified Eagle’ Medium (DMEM), and chondrocytes were obtained by overnight digestion of the cartilage with 0.5% collagenase-A diluted in DMEM containing 1% foetal calf serum (FCS) at 37°C.

2.2.4.2 Chondrocyte Viability Count

Viability of isolated chondrocytes was estimated using the trypan blue exclusion test. An equal volume of 0.5% trypan blue was added to the cell suspension. Viable cells were counted under a light microscope. Viable cells appeared bright white, while dead cells were dark blue. Cell viability for all fresh chondrocyte preparations was greater than 95%.

2.2.4.3 Chondrocyte 3-D Pellet Culture

Chondrocytes were cultured in 3-D pellets using a modification of the method described by Kato et al., (1988). Briefly, 10^5 freshly isolated, monolayer-passaged or thawed chondrocytes (Fig. 2.4) were resuspended in 1 ml DMEM containing gentamicin (50 µg/ml), amphotericin B (2.5 µg/ml), L-glutamine (300 µg/ml), L-ascorbic acid (50 µg/ml) and 10% FCS or 10% horse serum (HS) in 15 ml polypropylene conical tubes. The cells were centrifuged for 5 minutes at 67 x g to form aggregates. The tubes were maintained at 37°C in a humidified CO₂ incubator with the caps of the tubes closed. After 48 hours, the cells formed cohesive disc-shaped pellets, which could be manipulated with forceps. The medium was changed
every second day after the first three days of incubation, and fresh ascorbic acid was added to the medium with every change. Pellets were collected at various intervals from 0 to 35 days, depending on experimental requirements. For some experiments, TGF-β1 (10 ng/ml) or T3 (100 ng/ml) was included in the medium from day 7 until day 28. In some experiments, staurosporine (1 µM) was added to the medium at day 20, and the pellets were harvested after 6 and 24 hours. For light and electron microscopy, pellets were rinsed with 0.1 M cacodylate buffer at room temperature, fixed in cold 2.5% glutaraldehyde/4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 24 hours at 4°C and processed for light and electron microscopy as described in section 2.2.2.
Figure 2.4 Summary of the different processes carried out using isolated chondrocytes in this study.

Chondrocytes were isolated by overnight collagenase digestion of cartilage. Some isolated cells were cultured directly as a pellet. Chondrocytes not used in pellet culture were frozen or cultured in a monolayer. Thawed chondrocytes were cultured as pellets or cultured first in a monolayer then as pellets. Monolayer cultures were trypsinised and cultured as pellets or frozen.
2.2.4.4 Chondrocyte Monolayer Culture

Freshly isolated or frozen chondrocytes (5 x 10^5) were cultured in 25 cm^2 tissue culture flasks in an incubator at 37°C and 5% CO_2 in the presence of either 10% FCS or 10% HS. The medium was changed every 2 days. In some experiments at day 7, the confluent cells were trypsinised with trypsin-EDTA and cultured as pellets. In other experiments, the confluent cells were incubated in 0.1% FCS for 24 hours, then in the presence or absence of 1 μM staurosporine for 24 hours, then trypsinised and centrifuged; the pellets were processed for light and electron microscopy.

2.2.4.5 Chondrocyte Storage in Liquid Nitrogen

Freshly isolated or trypsinised monolayer-cultured chondrocytes (10^6) were resuspended in a 1 ml freezing medium consisting of DMEM, 10% FCS and 10% dimethyl sulphoxide (DMSO). The cells were gradually cooled by the rate of -1°C/minute using “Mr. Frosty” apparatus, kept for 24 hours at -80°C and stored in liquid nitrogen (-196°C) for a minimum of 3 months. For use in culture, frozen chondrocytes were quickly thawed in a 37°C water bath, expanded in monolayer and/or cultured as pellets. The viability of frozen chondrocytes was less than 50% for cells isolated from foetal and greater than 75% for cells isolated from postnatal horses.

2.2.4 Preparation of Cryosections

Deminerlised tissue samples or non-demineralised cultured pellets were collected, washed in PBS, fixed in 4% paraformaldehyde for 30 minutes and infiltrated with 25% (w/v) sucrose in PBS for 2 hours at 4°C. Samples were dried on absorbent filter paper, embedded in tissue-freezing medium (OCT compound), snap-frozen in liquid nitrogen and stored at –80°C. Cryosections (5-10 μm thickness) were cut on a microtome in a cryostat (Leica CM 1900) and collected on 3-amino-propyl-tri-ethoxy-silane (TESPA)-coated glass slides. Slides were stored at –80°C until used.
2.2.5 Haematoxylin and Eosin Staining

Cryosections were immersed in 70% ethanol for 10 seconds, then washed in distilled water for 10 seconds. Sections were stained with Meyer’s haematoxylin for 60 seconds, placed in tap water for 10 seconds and stained with 70% ethanol for 60 seconds. After being dehydrated in ascending grades of ethanol (70, 95 and 100%) for 10 seconds each, sections were cleared in xylene for 30 seconds and mounted with DPX.

2.2.6 Cathepsin B Immunohistochemistry

Cathepsin-B localisation in equine chondrocyte pellet culture was carried out using a sheep anti-human cathepsin B antibody (kindly provided by Dr. Elizabeth Davies, School of Veterinary Medicine, University of Cambridge, UK). Cross reaction of the antibody between human and equine species has been confirmed (Glaser et al., 2003).

Cryosections were washed in PBS for 5 minutes. Endogenous peroxidase was inactivated by incubation of sections with 0.3% hydrogen peroxide (H₂O₂) in methanol for 20 minutes. Non-specific reactions were blocked by treatment with 3% bovine serum albumin (BSA) in PBS containing 10% FCS for at least 30 minutes at room temperature. Sections were incubated with sheep anti-human cathepsin B antibody (1:400) for 1 hour at room temperature, while the control sections were incubated with the same dilution of normal sheep serum. Biotinylated rabbit anti-sheep immunoglobulin (10 µg/ml in PBS) was added to the sections as a secondary antibody for 1 hour at room temperature, followed by incubation with Vectastain Avidin Biotin Complex (ABC) solution for 1 hour at room temperature. The antibody was visualised by treatment of sections with diaminobenzidine tetrahydrochloride (1mg/ml; DAB) in PBS containing 0.2% H₂O₂. After the specific reaction appeared (3-5 minutes), the DAB was removed and the sections were washed with PBS several times, and mounted with Aquamount. Brown intracytoplasmic granules appeared in positively stained cells.
2.2.7 Histochemical Localisation of Alkaline Phosphatase

Alkaline phosphatase activity was detected in cryosections prepared from chondrocyte pellet culture using an Alkaline Phosphatase Detection Kit, which is a colorimetric assay using p-nitrophenol phosphate as a substrate. Sections were stained according to the manufacturer’s procedures with modifications. Briefly, sections were washed in PBS and incubated in alkaline-dye mixture at room temperature for 20 minutes with sections protected from light during the reaction time. Slides were washed in PBS and counterstained with a nuclear stain (“Neutral Red Solution, Buffered”), which was diluted in PBS (1:50). Sections were air-dried overnight and mounted with DPX. Blue granules appeared in positive cells.

2.2.8 Detection of Calcium Deposition

Cryosections were stained according to Von Kossa’s method. Briefly sections were incubated with 1% silver nitrate solution and exposed to a bright light for 60 minutes. After being washed in PBS, sections were flooded with 2.5% sodium thiosulphate for 5 minutes at room temperature, washed in PBS, counterstained with Mayer's haematoxylin for 1 minute and mounted with Aquamount. Calcified ECM appeared as dark brown to black areas.

2.2.9 Histomorphometric Analysis of Pellets

Histomorphometric parameters related to pellet growth such as pellet thickness and cellular density were analysed, and the percentage of different types of chondrocytes was calculated. Measurements were carried out on images of semi-thin sections stained with methylene blue. Images were captured with a digital camera linked to an Olympus BX 60 Microscope. All pellets took on the shape of the bottom of the tube, thus all sections had a shape similar to that shown in Fig. 2.5. Pellet thickness was defined as the distance between the top and bottom of semi-thin sections, and the mean thickness of each pellet was calculated from 3 locations (Fig. 2.5A). Cellular density was defined as the total number of cells per specific area, and the mean cellular density was calculated from 6 locations per pellet (Fig. 2.5B). Different forms of chondrocytes were counted from 6 locations per pellet (Fig. 2.55B), and expressed as a percentage of the total cell number. Pellet thickness and
cellular density measurements were made with Image-Pro Plus software and dying chondrocyte counts were carried out manually. Measurement of pellet thickness was made using a 20x objective and measurement of cellular density was made using a 40x objective, while cell counting was carried out using a 100x objective.

Fig. 2.5 Locations of measurements taken from pellet semi-thin sections. A: Pellet thickness (interrupted lines). B: Cellular density and dying chondrocyte counts (circles).
2.2.10 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

2.2.10.1 Isolation of Total RNA
Pellets were snap-frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated using a combination of Tri Reagent and the SV RNA Isolation Kit. Tri-Reagent (1 ml) was added to the frozen samples, which were homogenised using a Kinematica Polytron Homogeniser. The homogenate was transferred to 1.5 ml microcentrifuge tubes, and left to stand at room temperature for 30 minutes. Chloroform (300 µl) was added to each sample then samples were vortexed for 15 seconds, left to stand at room temperature for 5 minutes and centrifuged at 15120 x g at 4°C for 15 minutes. Three layers were formed; a superficial aqueous layer containing RNA, a middle white layer containing protein and a deep pink layer containing DNA. The aqueous layer was transferred into a new 1.5 ml microcentrifuge tube and an equal volume of RNA Binding Solution (RBS; a mixture of equal volumes of SV Lysis Buffer and 90% ethanol) was added to each tube. RNA/RBS mixture (700 µl) was transferred to columns supplied with the kit and centrifuged at 13148 x g for 1 minute at room temperature, and the flowthrough was discarded. SV Wash solution was used to wash the samples on the column. The RNA, which was adsorbed onto the filter, was treated with 5 µl DNase solution and the columns were left at room temperature for 15 minutes. The samples were washed with DNase stop solution (200 µl) then twice with SV Wash Solution before being transferred to fresh 1.5 ml microcentrifuge tubes. RNA was eluted in 55 µl diethylpyrocarbonate (DEPC)-treated water. The amount of RNA was calculated as 40 µg/ml per absorbance unit at 260 nm using a spectrophotometer. The quality of the RNA was evaluated by measuring the ratio of absorbance at 260 nm and 280 nm; RNA was judged to be of acceptable purity when the 260/280 ratio was approximately 2.0. RNA samples were stored at -80°C.

2.2.10.2 First Strand cDNA Synthesis
First strand complementary DNA (cDNA) was synthesised using Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase (200 U/µl) according to the manufacturer’s instructions. Briefly, a mixture of 1 µg RNA, 0.5 µg oligo dTs (3’-
TTTTTTTTT-5’), 1.25 µl 10 mM deoxynucleotide triphosphate (dNTPs; dATP, dCTP, dGTP and dTTP) and DEPC-treated water was added to a total volume of 12 µl. This mixture was heated to 65°C for 5 minutes and immediately placed on ice for at least one minute. A mixture of 5 µl 5x M-MLV reaction buffer, 7 µl DEPC-treated water and 1 µl M-MLV reverse transcriptase was added to each sample. Negative controls containing no reverse transcriptase were set up in parallel. Samples were incubated for 1 hour at 42°C and cDNA was stored at -80°C.

2.2.10.3 Polymerase Chain Reaction (PCR)

PCR was carried out to test the successful synthesis of cDNA, to optimise PCR conditions for aggrecan, and to investigate expression of collagen type II, collagen type X, and aggrecan in pellet cultures. Ribosomal protein S23 (RP-S23), the primers for which were optimised for equine sequences by Dr. Michiko Mirams (School of Veterinary Science, University of Melbourne) was used as a positive control. To examine the expression of the gene of interest, a mixture of 10x Taq DNA polymerase reaction buffer (2.5 µl), 25 mM MgCl2 (0.76 µl), 2.5 mM dNTPs (0.5 µl), 20 µM forward primer (0.5 µl), 20 µM reverse primer (0.5 µl), Taq polymerase (5 U/µl; 0.2 µl) and cDNA (2 µl) was used. The negative control was the same mixture but instead of cDNA, distilled water (2 µl) was added. PCR was carried out using a thermal cycler. Thermal cycling consisted of 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds.

2.2.10.4 Agarose Gel Electrophoresis

Agarose gel (1%) in 1x Tris base, boric acid and EDTA (TBE; 89 mM Tris base, 89 mM boric acid and 2.5 mM EDTA) was used. Ethidium bromide (1 ml; 0.5 µg/ml) was added to 40 ml dissolved gel. After solidification, the gel was transferred into a gel tank filled with 1x TBE buffer. Samples (5 µl) were mixed with 10x loading buffer [(1 µl) 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% (w/v) sucrose, 10 mM Tris-HCL (pH 8.0), 2% (w/v) sodium dodecyl sulphate (SDS) and 1 mM EDTA] and added into the wells of the gel. DNA was
electrophoresed using a 100V potential difference for about 80 minutes. PCR products were visualised under a UV transilluminator and photographed using a digital camera and Kodak 1D 2.0 software. Alternatively, SYBER Safe fluorescent gel stain was used instead of ethidium bromide (with 0.5% TBE instead of 1% TBE buffer). SYBER safe fluorescent gel images were captured using an image acquisition system, Chemi-Smart 2000.

### 2.2.11 Optimisation of Conditions for Aggrecan PCR

Aggrecan PCR primers based on a partial equine sequence were designed using the software provided by the Whitehead Institute for Biomedical Research (http://frodo.wi.mit.edu/cgi-bin/primer3cgi). PCR reactions were carried out under different conditions, with different concentrations of MgCl₂, dNTPs and primers (Table 2.2). Among the different conditions, conditions “A” and “B” showed a band of appropriate size, 96 base pairs, and condition “B” was selected for sequencing.

**Table 2.2: Different conditions used for aggrecan PCR optimisation.**

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### 2.2.12 Sequencing

Aggrecan DNA was purified from the PCR product using UltraClean PCR Clean–up Kit following the manufacturer’s instructions. Purified PCR product was prepared for sequencing using BigDye Terminator V3.1. A sequencing reaction (12
µl) consisting of 2.5 µl of 20 µM forward or reverse primer, 4 µl BigDye Terminator V3.1 Master Mix and 27 ng DNA template was made. PCR was carried out using a thermal profile as follows: 94°C for 4 minutes, and 40 cycles of 90°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. A mixture of 95% ethanol (62.5 µl), 3 M sodium acetate pH 4.6 (3 µl) and distilled water (14.5 µl) was added to the PCR product (12 µl), to precipitate DNA. The aggrecan PCR product was vacuum-dried and sent to the Australian Genome Research Facility (AGRF) for sequencing by the capillary separation method. The resulting sequencing file was analysed using Edit–View software. To confirm the identity of the sequenced products as aggrecan, a further analysis was carried out using Basic Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.13 Real-Time Quantitative PCR (Q-PCR)

All primers used in this study except aggrecan and MMP-13 were designed and optimised for equine sequences by Dr Michiko Mirams, School of Veterinary Science, University of Melbourne (Table 2.3). A published sequence of human MMP-13 (Lafleur et al., 2005), optimised for use with equine material by Dr. Mirams, was used in this study.

Q-PCR is a quantitative technique used to measure progress occurring through PCR amplification in real time. In this technique a fluorescent reaction is detected every cycle. The cycle at which the fluorescence generated from the DNA amplification exceeds the background fluorescence is called the cycle threshold or “CT”. The greater the initial transcript number, the earlier the PCR product will amplify, so the lower the CT.

Among the different fluorescent dyes used for Q-PCR, SYBER green, is the easiest and cheapest, therefore it was chosen for this study. SYBER green binds to every double strand of DNA giving fluorescent signals. A passive reference fluorescence dye, ROX, was used to correct for artefacts in signal measurements coming from non-specific reactions. Using a Stratagene MX300P Q-PCR machine, it is possible to compare CTs from different templates.
A primer set is defined as efficient if it allows a template to duplicate every cycle; an efficiency of 90-110% is considered adequate for Q-PCR analysis. To calculate the efficiency for aggrecan primers, serial dilutions of chondrocyte pellet-derived cDNA (1:1, 1:10, 1:100 and 1:1000) and aggrecan primers (400 nM-700 nM) were used in Q-PCR reactions. Under optimal conditions with a primer concentration of 700 nM, the efficiency was 92.5%.

To study changes in gene expression in pellet cultures, RNA was extracted from pellets (3 pools of at least 10 pellets each for each treatment) treated under a variety of conditions. A mixture (19 μl) of 0.1 μl ROX, 10 μl 2x Platinum SYBR green Supermix-UDG, and primers at concentrations of 500-900 nM (Table 2.4) was made up. cDNA (1 μl) was added to each well of the 96-well plates of the Q-PCR machine, and 1 μl Milli-Q water (water purified on a Millipore Q system) was added to the negative controls. The Q-PCR reaction was carried out with thermal cycles as follows: 1 cycle: 95°C for 5 minutes, 40 cycles: 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 1 cycle 95°C for 1 minute, 65°C for 30 seconds and 95°C for 30 seconds. Results were analysed using the relative expression software tool (Pfaffl et al., 2002). This software uses the pair-wise randomisation tests to compare the expression of the gene of interest to that of the housekeeping gene, RP-S23. Randomisation tests with a pair-wise reallocation were used because it is considered as the most appropriate statistical method for relative gene expression measurement. The housekeeping gene, RP-S23, was selected because its expression levels in proliferative and hypertrophic zones of growth plate are not different (Wang et al., 2004). The expression of RP-S23 did not differ with any of the experimental conditions investigated. Expression of genes of interest in experimental samples was normalised to RP-S23 expression and expressed relative to expression of genes of interest in control samples (normalised to RP-S23 expression).

2.2.14 Statistical Analysis

For all analysis other than Q-PCR, statistical differences between groups were evaluated using one way ANOVA. For pellet weight measurements, the mean was taken from 4 pellets. For other analysis, measurements were taken from 3 replicates. For each comparison, a P-value of less than 0.05 was considered to be significant.
**Table 2.3 Primer sequences for different genes used in PCR studies.**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>*Forward /Reverse</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>RP-S23</td>
<td>1-Forward</td>
<td>CAGAGTGGCAGTCAAGCAG</td>
</tr>
<tr>
<td></td>
<td>2-Reverse</td>
<td>CACACTTACCTGATGGCAGAA</td>
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<td>Collagen type-I</td>
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<td>Collagen type-II</td>
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<td></td>
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<td>CGCGGCTGGTACTTCTAAT</td>
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<td></td>
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<td>ACCGTCTGGATGGTGATGTC</td>
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* Numbers refer to exons the primers are located in.*
Table 2.4 Primer concentrations used in Q-PCR reactions.

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<tr>
<th>Forward/Reverse Primer</th>
<th>Final Concentration (nM)</th>
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<td>CTGF</td>
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<tr>
<td>VEGF</td>
<td>500</td>
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<tr>
<td>FGFR3</td>
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<td>MMP-13</td>
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<td>Aggrecan</td>
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### Appendix 2.1 Sources of Chemicals.

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<tr>
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<tr>
<td>Aquamount</td>
<td>BDH</td>
<td>London, UK</td>
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<tr>
<td>BigDye Terminator V3.1</td>
<td>Applied</td>
<td>Buckinghamshire, UK</td>
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<tr>
<td>Biotinylated rabbit anti-sheep immunoglobulin</td>
<td>Pierce</td>
<td>Rockford, USA</td>
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<tr>
<td>Boric acid</td>
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<td>Darmstadt, Germany</td>
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<tr>
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<td>Collagenase A</td>
<td>Roche</td>
<td>Basel, Switzerland</td>
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<td>DAB</td>
<td>DAKO Corporation</td>
<td>Carpinteria, CA, USA</td>
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<tr>
<td>DMEM</td>
<td>Invitrogen</td>
<td>Carlsbad, California, USA</td>
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<td>DMSO</td>
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<td>Madison, WI, USA</td>
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<td>DPX</td>
<td>ProSciTech</td>
<td>Thuringowa, Australia</td>
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<td>EDTA</td>
<td>Ajax Finechem</td>
<td>Seven Hills, Australia</td>
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<td>FCS</td>
<td>Invitrogen</td>
<td>Carlsbad, California, USA</td>
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<td>Gentamycin</td>
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<td>Carlsbad, California, USA</td>
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<td>Gluteraldehyde</td>
<td>ProScitech</td>
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<td>BDH</td>
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<td>Merck</td>
<td>Darmstadt, Germany</td>
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<td>Invitrogen</td>
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<td>L-glutamine</td>
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<td>MgCl$_2$ (25mM)</td>
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<td>M-MLV reverse transcriptase 200 U/µl</td>
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<td>Address</td>
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<td>Alkaline Phosphatase Detection Kit</td>
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<td>UltraClean PCR Clean-up Kit</td>
<td>Biocompare</td>
<td>San Francisco, USA</td>
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<td>Vectastain ABC Kit</td>
<td>Vector Laboratories</td>
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### Appendix 2.3 Sources of Tissue Culture Plasticware, Specialised Equipments and Software.

<table>
<thead>
<tr>
<th>EQUIPMENT/ SOFTWARE</th>
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<th>ADDRESS</th>
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<tr>
<td>15 ml Polyporolyne Conical Tubes</td>
<td>Becton Dickinson Labware</td>
<td>Franklin Lakes, NJ, USA</td>
</tr>
<tr>
<td>Chamber Slides</td>
<td>Lab-Tek</td>
<td>Rochesrer, NY, USA</td>
</tr>
<tr>
<td>Chemi-Smart 2000 gel documentation system</td>
<td>Vilber Lourmat</td>
<td>Marne-la-Vallée Cedex 1, France</td>
</tr>
<tr>
<td>Freezing Container (Mr. Frosty)</td>
<td>Nalgene Labware</td>
<td>Rochester, NY, USA</td>
</tr>
<tr>
<td>Image-Pro Plus Software</td>
<td>Media Cybernetics</td>
<td>Silver Spring, MD, USA</td>
</tr>
<tr>
<td>Kinematica Polytron Homogeniser</td>
<td>Kinematica</td>
<td>Luzern, Swizerland</td>
</tr>
<tr>
<td>Kodak 1D 2.0 Software</td>
<td>Kodak</td>
<td>Rochester, NY, USA</td>
</tr>
<tr>
<td>Leica CM 1900 Cryostat</td>
<td>Leica</td>
<td>Wetzlar, Germany</td>
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<td>Reichert OmU12 Ultramicrotome</td>
<td>Reichert-Jung Ag</td>
<td>Vienna, Austria</td>
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<tr>
<td>Stratagene MX3000P Real-Time PCR Machine</td>
<td>Stratagene</td>
<td>La Jolla, USA</td>
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<tr>
<td>Tissue Flasks</td>
<td>Becton Dickinson Labware</td>
<td>Franklin Lakes, NJ, USA</td>
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CHAPTER 3: PHYSIOLOGICAL CELL DEATH IN EQUINE GROWTH CARTILAGE

3.1 Introduction

The foetal skeleton is composed largely of cartilage, which is gradually replaced by bone (except at the articular surface) by the process of endochondral ossification. During endochondral ossification, the chondrocytes in growth cartilage undergo proliferation and hypertrophy. Death of hypertrophic chondrocytes is an obligatory step in the pathway of endochondral ossification. Early ultrastructural studies have described two types of hypertrophic chondrocytes, dark and light cells, however, it was not clear from these studies whether light and dark cells are separate populations, or different states of differentiation of a single cell population (Wilsman et al., 1981). Furthermore, the early studies described the disintegration of hypertrophic chondrocytes but they did not define the mechanisms by which they die (Farnum and Wilsman, 1987). No further mention of light and dark chondrocytes was made in subsequent publications. In recent years, many authors have reported apoptosis to be the fate of hypertrophic chondrocytes on the basis of detection of DNA strand breaks using the TUNEL method, or other molecular features known to be associated with apoptosis (Adams and Shapiro, 2002; Shum and Nuckolls, 2002). Cells dying by apoptosis have an unmistakable morphological appearance including nuclear condensation into geometric shapes and formation of apoptotic bodies (Kerr et al., 1972). Recently, hypertrophic chondrocytes have been described as dying by forms of PCD morphologically distinct from apoptosis, and different terminologies have been used (Roach and Clarke, 1999; Roach and Clarke, 2000; Roach et al., 2004; Shapiro et al., 2005). Although many studies on endochondral ossification and cell death have been undertaken (Farnum and Wilsman, 1987; Farnum and Wilsman, 1989; Farnum et al., 1990; Hatori et al., 1995; Erenpreisa and Roach, 1999; Rajpurohit et al., 1999; Dunker et al., 2001; Doi et al., 2002; Donohue and Demay, 2002; Dunker et al., 2002; Ploumis et al., 2004; Bluteau et al., 2006; Trepeczik et al., 2006), it is difficult to obtain from these published data a clear understanding of the mechanisms of hypertrophic chondrocyte death. Studying chondrocyte death is important not only for understanding the process of
endochondral ossification, but also for understanding the pathogenesis of orthopaedic diseases affecting horses, such as osteochondrosis. The aim of the current study was to document the structure of growth cartilage throughout development and growth of the equine skeleton, with particular emphasis on the morphology of dying hypertrophic chondrocytes.

3.2 Results

3.2.1 Histology of Equine Growth Cartilage during Foetal and Postnatal Development

Cryosections and semi-thin sections from growth cartilage of the head of the humerus during different stages of development including foetal and postnatal stages were examined by light microscopy. Table 3.1 summarises the grossly visible features of the different specimens examined. At 3 months post-conception, before formation of the SOC, the head of the humerus was completely cartilaginous; this cartilaginous structure is referred to here as the chondroepiphysis and contained growth cartilage (PGC) giving rise to the POC (Fig. 3.1A). By 8 months post-conception, the SOC had appeared, dividing the chondroepiphysis into two parts. There was now growth cartilage beneath the prospective articular cartilage (AEGC). Between the SOC and POC, there were two regions of growth cartilage, the “reverse growth plate” facing the SOC, and the PGC facing the POC, as shown in a section from a 9-month foetus in Fig. 3.1B. By 6 months of age, the cartilage was completely replaced by bone except at two locations, the articular surface and the physis. There was still a small amount of AEGC beneath the permanent articular cartilage (Fig. 3.1C). The reverse growth plate had been replaced by bone, but there was still a substantial amount of PGC (Fig. 3.1D).

The chondrocytes in all of the regions of growth cartilage were organised into three morphologically distinct zones: resting, proliferative, and hypertrophic (Fig. 3.1). Resting chondrocytes were rounded or oval and randomly distributed in the ECM (Fig. 3.2A). Proliferative chondrocytes were flattened to oval in shape and arranged in column-like structures parallel to the longitudinal axis of the bone (Fig. 3.2B). Hypertrophic chondrocytes were large elliptical, oval or irregular-shaped cells; some of them appeared to completely fill their lacunae and some did not (Fig.
3.2C). A small number of lacunae of the hypertrophic zone, close to the cartilage-bone interface, appeared empty or contained only cell debris (Fig. 3.3).

Cartilage canals were observed in the foetal growth cartilage (Fig. 3.1 A, B) but were completely absent from the postnatal samples examined (Fig. 3.1C, D). The contents of the cartilage canals varied with age. During early foetal development (between 3 and 6 months), cartilage canals were simply structured from layers of connective tissue containing flattened cells (Fig. 3.4A, B) and sometimes contained thick-walled arterioles (Fig. 3.4B). By 8 to 9 foetal months, the canals contained many cell types. In addition to red blood cells (RBCs) in blood vessels, undifferentiated mesenchymal cells with the characteristic large pale nucleus and little cytoplasm were predominantly present (Fig. 3.4C). Cells of the cartilage canals involved in initiation of the SOC differentiated into osteoblasts and osteoclasts (Fig. 3.4D)

3.2.2 Physiological Death of Hypertrophic Chondrocytes

Ultrastructural studies were conducted on specimens from PGC and AEGC obtained from the humerus of foetal (3, 6, 9 and 10 month) and postnatal (6 month) horses. In all specimens of growth cartilage examined, two types of chondrocytes could be identified within the proliferative and hypertrophic zones: dark and light chondrocytes (Fig. 3.5). Dark chondrocytes were characterised by a dark nucleus and electron-dense cytoplasm containing well developed and often dilated RER (Fig. 3.6A-C, Fig. 3.7A, Fig. 3.8 A, Fig. 3.9A, Fig. 3.10 A, Fig. 3.11A, B, Fig. 3.12A) and inconspicuous Golgi apparatus with numerous secretory vesicles (Fig. 3.9A, B, Fig. 3.13). Light chondrocytes, in contrast, were characterised by electron-lucent cytoplasm containing sparse cisterns of RER, less developed Golgi apparatus and very few secretory vesicles, and their nuclei contained primarily euchromatin (Fig. 3.6D-F, Fig. 3.7B, Fig. 3.8B, Fig. 3.9C, D, Fig. 3.10B, Fig. 3.11B, Fig. 3.12B, Fig. 3.14A, B). In the zone of chondrocyte hypertrophy, cells with mixed electron-dense and electron-lucent cytoplasm were never seen. In all specimens examined, with increasing proximity to the ossification front, both types of chondrocytes appeared to be undergoing progressive changes ending with their disintegration. Dark cells appeared to undergo progressive extrusion of their cytoplasm with secretory contents
into the ECM (Fig. 3.9A, Fig. 3.15A-D,) and their nucleus underwent patchy condensation (Fig. 3.6A-C, Fig. 3.8A, Fig. 3.11C, Fig. 3.15A, C). Light cells appeared to disintegrate within the cell membrane (Fig. 3.6D-F, Fig. 3.8B, 3.14A, B,) and also nuclear condensation was prominent in later stages (Fig. 3.11D). The different types of morphology of light and dark hypertrophic chondrocytes at different positions within growth cartilage (as illustrated in Fig. 3.5) were seen in all specimens examined, whether from foetal or postnatal growing horses, and whether from PGC (Fig. 3.5, Fig. 3.6, Fig. 3.7, Fig. 3.8, Fig. 3.11, Fig. 3.13, Fig. 3.14, Fig. 3.15) or AEGC (Fig. 3.9, Fig. 3.10, Fig. 3.12).

Cells with similar morphology to those presented in Fig. 3.6A (‘early dark’), Fig. 3.6C (‘dying dark’), Fig. 3.6D (‘early light’) and Fig. 3.6F (‘dying light’) were counted in semithin sections of the PGC and AEGC (where present) of all specimens on which histology was performed. Cell counts were taken from two regions of the growth cartilage; one region was defined as the mid-point, and was half-way between the last few flattened proliferative cells and the ossification front, and the second region was the last three lacunae before the ossification front (Fig. 2.3). All cells were judged as being in one of the above four categories on the basis of the following criteria. Cells ‘A’ were characterised by an electron-dense cytoplasm, a cytoplasmic/nuclear ratio of more than one and many cytoplasmic processes. Cells ‘C’ were characterised by an electron-dense cytoplasm, a cytoplasmic/nuclear ratio of less than one, and very few or no cytoplasmic processes. Cells ‘D’ were characterised by an electron-lucent cytoplasm and the presence of no or very few empty cytoplasmic spaces, and the cells ‘F’ were characterised by an electron-lucent cytoplasm and extensive cytoplasmic digestion recognisable as many empty spaces.

For both PGC and AEGC, the percentage of dark or light chondrocytes that were classified as ‘early’ was higher at the mid-point of the growth cartilage than at the last three lacunae before the ossification front; the percentage of dark or light chondrocytes that were classified as ‘dying’ was significantly higher in the last three lacunae than at the mid-point (Fig. 3.16). When the data for animals with both PGC and AEGC (i.e. animals of 9 months and older) were re-arranged so as to compare the total number of early and dying dark or light cells between PGC and AEGC, the percentage of dark chondrocytes was seen to be higher in the PGC than in the AEGC...
both at the mid-point and in the last few lacunae (Fig. 3.17). Conversely, the percentage of light chondrocytes was lower in the PGC than in the AEGC. Moreover, the total numbers of early and dying dark or light cells were not significantly different between the mid-point and the last three lacunae, for either PGC or AEGC, that is, the ratio of dark : light cells did not change between the mid-point and the last three lacunae (Fig. 3.17).

It is interesting to note that cells showing the morphology of those described as paralysed (Roach and Clarke, 1999), were seen around the cartilage canals in the head of the humerus of a 3-month equine foetus (L. Tatarczuch School of Veterinary Science, University of Melbourne).
Table 3.1 Timing of presence of POC, SOC and cartilage canals in the specimens of equine head of humerus examined in this study.

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<tr>
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</tr>
<tr>
<td>8 month foetal</td>
<td>+</td>
<td>-</td>
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<td>9 month foetal</td>
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<tr>
<td>10 month foetal</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6 month postnatal</td>
<td>+</td>
<td>+</td>
<td>-</td>
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</table>
Figure 3.1: Morphology of equine growth cartilage from the proximal end of the humerus during foetal (A, B) and postnatal (C, D) development.

A: Chondroepiphysis from a 3-month foetal foal before formation of the SOC.
D: PGC from a 6-month postnatal horse.

Arrowheads indicate the articular surface, and arrows in A, B indicate cartilage canals. Note resting (R), proliferative (P) and hypertrophic (H) zones. Bar in parts A, C, D = 285 μm, and in part B = 650 μm.
Figure 3.2 Zones of growth cartilage from a 6-month equine foetus.
Section of chondroepiphysis from a 6-month foetal foal. Resting (A), proliferative (B) and hypertrophic (C) zones. Arrow indicates the articular surface. Note POC. All parts have the same magnification; bar = 55 μm.
Figure 3.3 Hypertrophic zone of equine foetal growth cartilage.
Semi-thin section of the head of the humerus of a 6-month foetus. Arrows indicate lacunae that appear empty. Note POC. Bar = 10 μm.
Figure 3.4 Morphology of cartilage canals in equine foetal growth cartilage.
Semi-thin sections from growth cartilage of the proximal end of the humerus from equine foetuses 3 (A), 5 (B), 8 (C) and 9 (D) months post-conception. Arrows indicate the flattened connective tissue layers surrounding the cartilage canals (A, B), mesenchymal cells (C) and osteoclasts in D. Arrowheads indicate an arteriole in B and osteoblast (D). Bars = 10 μm in A, C, D and 80 μm in B.
Figure 3.5 Morphology of chondrocytes in growth cartilage from the physis of the humerus of a 10-month equine foetus.

A: Semi-thin section; labels B-E indicate the regions corresponding to micrographs shown in parts B-E. B-E: Electron micrographs showing light (li) and dark (da) chondrocytes in the late proliferation zone (B), early hypertrophic zone (C), middle hypertrophic zone (D) and late hypertrophic zone near the ossification front (E). Bar = 25 µm in A. Parts B-E have the same magnification; bar = 10 µm.
Figure 3.6 Morphology of dying hypertrophic chondrocytes.

Electron micrographs of the proximal physis of the humerus of a 3-month equine foetus. A-C: Hypertrophic dark chondrocytes in the early (A), middle (B), and late (C) zones of hypertrophy corresponding to parts C, D and E of Fig. 3.5. The nucleus (small arrows) shows progressive patchy chromatin condensation and the cytoplasm appears to undergo progressive extrusion (arrowheads) into the ECM until there is almost none left (C). D-F: Hypertrophic light chondrocytes in the early (D), middle (E), and late (F) zones of hypertrophy. The nucleus (small arrows) remains intact and apparently functional while the cytoplasm disintegrates within an intact cell membrane (arrowhead). Note RER (large arrows). The magnification is the same in all parts; bar = 2 µm.
Figure 3.7 Electron micrographs from the physis of the humerus of a 3-month equine foetus.

Higher magnification of hypertrophic dark (A) and light (B) chondrocytes in the early zone of hypertrophy. Arrows indicate RER. Magnification is the same in both parts; bar = 0.5 µm.
Figure 3.8 Electron micrographs from the physis of the humerus of a 6-month equine foetus.

Hypertrophic dark (A) and light (B) chondrocytes from close to the ossification front. Arrows indicate nucleus and arrowheads indicate RER. Parts A, B have the same magnification; bar = 2 μm.
Figure 3.9 Electron micrographs from the AEGC of the humerus of a 9-month equine foetus.

Hypertrophic dark (A, B) and light (C, D) chondrocytes from the early hypertrophic zone. Arrowheads in A indicate cytoplasmic extrusions. Arrows in A-D indicate RER. In part B, note many secretory vesicles (sv). Parts A, C have the same magnification; bar = 4 μm. Parts B, D have the same magnification; bar = 1.5 μm.
Figure 3.10 Electron micrographs from the AEGC of the humerus of a 10-month equine foetus.

Arrows indicate RER in hypertrophic dark (A) and light (B) chondrocytes. Parts A, B have the same magnification; bar = 2 μm.
Figure 3.11 Electron micrographs from the physis of the humerus from a 6-month postnatal foal.

A, C: Hypertrophic dark chondrocytes in the early (A) and late (C) zones of hypertrophy. B, D: Hypertrophic light chondrocytes in the early (B) and late (D) zones of hypertrophy. Arrows indicate RER in A, B and the nucleus in C, D. Magnification is the same in parts A, B, D; bar = 5 µm. Bar in C = 2.5 µm.
Figure 3.12 Electron micrographs from the AEGC of the humerus of a 6-month postnatal foal.
Hypertrophic dark (A) and light (B) chondrocytes from the early hypertrophic zone. Arrows indicate nucleus and arrowheads indicate RER. Parts A, B have the same magnification; bar = 2 μm.
Figure 3.13 Electron micrograph from the physis of the humerus of a 10-month equine foetus.
Higher magnification of a hypertrophic dark chondrocyte. Note abundant RER (arrow), well developed Golgi apparatus (arrowheads) and many secretory vesicles (sv). Bar = 0.4 μm.
Figure 3.14 Electron micrographs from the physis of the humerus of a 10-month equine foetus.

Hypertrophic light chondrocytes from close to the ossification front. Arrow indicates cell membrane, Arrowheads indicate RER. Areas of cytoplasmic digestion (S). Note nucleus (N). Bar = 2 μm in A and 0.5 μm in B.
Figure 3.15 Electron micrographs from the physis of the humerus of 10-month (A, B) and 9-month (C, D) equine foetuses.

Arrows indicate extrusion of cytoplasm of dark chondrocytes into ECM. Note many secretory vesicles (sv) in D. Bar = 4 μm in A, C, 1μm in B and 0.5 μm in D.
Figure 3.16 Dark and light hypertrophic chondrocyte counts in equine growth cartilage.

Early hypertrophic dark and light chondrocytes were counted in semi-thin sections of all specimens on which histology was undertaken (see Table 2.1) A: Counts from PGC. B: Counts from AEGC. Data represent mean ± SEM; n = 6 for PGC and n= 3 for AEGC. Significant differences between values are expressed as follows: a (P < 0.001), b (P < 0.01), c (P < 0.05) for comparison between the mid-point (MP) and the last three lacunae (LTL).
Figure 3.17 Comparison between dark and light hypertrophic chondrocyte counts in PGC and AEGC.

Data presented in Fig. 3.16 for animals with both PGC and AEGC were re-arranged to compare the number of dark and light hypertrophic chondrocytes in these two locations. The values for early and dying chondrocytes have been combined to give a single value for dark (or light) cells at the mid-point (or last three lacunae), for each of the PGC and AEGC. Data represent mean ± SEM; n = 3. Significant differences between values are expressed as a (P < 0.001) for comparison between PGC and AEGC. ns- no significant difference (for comparison between mid-point and last three lacunae).
3.3 Discussion

The aim of the current study was to clarify how the hypertrophic chondrocytes of equine growth cartilage die. Light microscopy of samples from different ages and stages of development showed that equine growth cartilage is similar to that in other species (Burkus et al., 1993; Rivas and Shapiro, 2002). Electron microscopic studies demonstrated that hypertrophic chondrocytes were identifiable as either light or dark as described for human (Hwang, 1978) and pig (Wilsman et al., 1981). Following proliferation, chondrocytes was present as either dark or light cells, and each cell type appeared to undergo morphologically different pathways of hypertrophy and death.

When discussing the significance of the distinctive morphology of light and dark chondrocytes, Wilsman et al. (1981) suggested as one possibility that they represent different stages of differentiation of one cell type. The observations made in the present study suggest that they represent two different post-proliferative populations, rather than different stages of a single population. This conclusion is based on the observation that light and dark cells were detectable from the end of the zone of proliferative chondrocytes to the ossification front. Moreover, at different levels of the growth cartilage, changes in the appearance of both light and dark cells were observed; these changes were consistent with the conclusion that each cell type undergoes morphologically different pathways of hypertrophy and death. These observations were supported by results from cell counting at different levels of the growth cartilage. Cell counts revealed that the total number of early and dying light or dark cells was similar in the mid-point and the last few lacunae of either PGC or AEGC. In addition, the proportion of cells identified as early dark or light cells was lower in the last few lacunae than at the mid-point of either PGC or AEGC. Conversely, the proportion of cells identified as dying dark or light cells was higher in the last few lacunae than at the mid-point of PGC or AEGC. Taken together, these observations support the hypothesis that dark and light hypertrophic chondrocytes are two different populations.

Because electron microscopy of tissue specimens is very time consuming, it was not possible to examine enough specimens to investigate whether there are differences in the proportion of light and dark chondrocytes with age, in such a way
that statistical conclusions could be made. The cell counts shown in Fig. 3.16 include data for either PGC or AEGC for all specimens in which the structure was present. The fact that the standard errors were small and that statistically significant differences were observed suggest that this was a valid approach to comparing the proportion of early and dying chondrocytes between different zones of growth cartilage at a specific anatomical location. The small standard errors also suggest that there are not likely to be substantial differences with age in the ratio of light : dark chondrocytes in either PGC or AEGC. The percentage of light chondrocytes was higher in the AEGC than in the PGC as previously described for porcine cartilage (Carlson et al., 1985). It is possible that these cells play different roles and may be secreting different products during the process of endochondral ossification.

It was observed that the dark (and to a lesser extent, light) chondrocytes contained well-developed RER suggesting that these cells are active in protein synthesis and secretion during the course of their death. Other authors have previously reported dark chondrocytes as highly active cells (Erenpreisa and Roach, 1999). Recently it was possible to demonstrate the involvement of both cell types in proteoglycan production using a specific stain for proteoglycans (results of L. Tatarczuch published in Ahmed et al., 2007). Matrix metalloproteinases-2, -9 and -13 as well as TGF-β have been reported to be included within the secretory vesicle components of hypertrophic chondrocytes (Marina et al 2001). The secretion may also include VEGF, an important angiogenic factor, since this is known to be expressed by hypertrophic chondrocytes (Gerber et al., 1999). Secretion of dark and light cells is likely to contain molecules important for cartilage maintenance and growth such as hyaluronan, which forms a layer surrounding hypertrophic chondrocytes to allow enlargement of their lacunae, and protect them from direct contact with matrix components (Pavasant et al., 1996).

It is well known that the usual pathway of protein secretion in many cell types includes three steps. First, formation of protein within the lumen of RER, then transportation of this protein into Golgi apparatus where the concentration, glycosylation and packaging into membranous vesicles occur, and finally release of these secretory vesicles from the cytoplasm by exocytosis (Eurell and Frappier, 2006). In the current study it was shown that the dark chondrocytes have well
developed RER and Golgi apparatus with numerous secretory vesicles, suggesting that the pathway of secretion is the same as described for other cell types. However, light chondrocytes appeared to have less developed Golgi apparatus and sparse secretory vesicles suggesting that light cells can secrete independently of the Golgi apparatus, at least for some products. A Golgi-independent mechanism of secretion has previously been reported in other cell types (reviewed by Nickel, 2003).

Morphological observations suggested that these two cell types die by different non-apoptotic mechanisms. The dark chondrocytes appeared to undergo extrusion of their cytoplasm into ECM, however, the light cells appeared to undergo cytoplasmic digestion within the cell membrane. It is generally accepted that death by apoptosis is a way to remove unwanted cells in different tissues followed by phagocytosis by specialised or adjacent cells without inducing inflammation. However, in cartilage phagocytosis is not possible due to the absence of such specialised phagocytic cells and the presence of chondrocytes within lacunae that are separated from each other by abundant ECM, making it difficult for these cells to be phagocytosed by the adjacent cells. It was observed in this study that some of the last lacunae before the ossification front appeared empty. It could be that the cells were out of the plane of the section, but in another study using serial sections from porcine growth cartilage, it has been shown that a small proportion of the terminal lacunae are indeed empty (Farnum and Wilsman, 1989). The presence of empty and closed lacunae close to the chondro-osseous junction indicates that these dying cells may digest themselves without phagocytosis by other cells. However, evidence has previously been provided for phagocytosis of cell remnants in open lacunae at the ossification front by macrophages and osteoclasts associated with the osteogenic invasion so this may assist in the removal of cell debris (Bronckers et al., 2000). Studies reporting apoptosis in growth cartilage relied on non-specific methods such as TUNEL staining, caspase activity detection, flow cytometry and gel electrophoresis (Ohyama et al., 1997). Furthermore some of the ultrastructural studies have shown typical dying dark chondrocytes, similar to those observed in the current study, as an example of apoptosis in growth cartilage (Zenmyo et al., 1996, page 432; Ploumis et al., 2004, page 500). Apoptotic cells were not seen in any of the specimens of growth cartilage examined in the current study. In a recent review, Roach et al.
(2004) introduced the term ‘chondroptosis’ to describe the death of dark chondrocytes and they showed similar pictures to dark chondrocytes shown in the current study, however they did not mention the death of light chondrocytes. More recently in a review by Shapiro et al. (2005), it has been suggested that hypertrophic chondrocytes undergo autophagic cell death. The authors suggested that due to the lack of nutrients and oxygen, the chondrocytes digest themselves to generate more ATP thus slowing their death. However, if that were the case one would expect to see more dying chondrocytes in the mid-point of the growth cartilage than close to the ossification front (where many blood vessels are present), and that is not the case, as dying chondrocytes were significantly more abundant close to the ossification front than in the upper layers of the hypertrophic zone. In addition, if the chondrocytes were dying by autophagic cell death, it would be possible to observe the increased number of double-membraned autophagosomes characteristic of this form of death, but that was not the case in either dark or light cells.

Apart from the morphological appearances of dying dark and light chondrocytes, the mechanisms of death of dark and light chondrocytes are not clear. Most of the studies describing the mechanism of chondrocyte death come from monolayer culture, and it is known that the chondrocytes do not undergo hypertrophy in such culture (see section 1.3.2). Very few studies have used intact cartilage to study the mechanism of chondrocyte death. It was reported that in equine articular cartilage explants caspase-9 inhibition significantly decreases chondrocyte death, described by the authors as apoptosis (Huser et al., 2006). However they showed cells (p1004; Fig. 1.B) typical of those described in the current study as dying dark chondrocytes. Thus caspases may be involved in death of hypertrophic dark chondrocytes. Bcl-2, a protein that protects against apoptosis, may play a role in survival of hypertrophic chondrocytes, since deletion of the gene encoding Bcl-2 leads to accelerated maturation of chondrocytes and shortening of long bones in mice (Amling et al., 1997). It would not be surprising if there is some overlap between the mechanisms regulating death of hypertrophic chondrocytes and those regulating apoptosis.

This study was conducted on normal specimens from foetal (3-10 months) and 6-month postnatal horses. It is still unclear if the hypertrophic chondrocytes in older
animals or in pathological conditions undergo the same modes of dark and light cell death.

In conclusion, the results presented here suggest that dark and light hypertrophic chondrocytes are two different populations of cells, and that they undergo morphologically distinct non-apoptotic modes of PCD.
CHAPTER 4: ESTABLISHMENT OF AN IN VITRO MODEL FOR STUDYING EQUINE CHONDROCYTE PCD

4.1 Introduction

In the previous chapter, the morphology of hypertrophic chondrocytes undergoing PCD in equine growth cartilage was documented. It was confirmed that there are two types of hypertrophic chondrocytes (dark and light cells) and observed that they appear to die by different non-apoptotic mechanisms.

Investigating the mechanisms of chondrocyte PCD has been difficult because there has been no in vitro model in which the isolated chondrocytes could be induced to die by PCD similar to that described in cartilage tissue (Roach et al., 2004). From extensive studies of chondrocyte monolayer culture, it has been shown that the expanded chondrocytes lose their characteristic round configuration, becoming fibroblast-like cells, and cease expression of chondrocytic markers such as collagen type II (Stewart et al., 2000; Malpeli et al., 2004). Furthermore, the chondrocytes in monolayer culture do not undergo hypertrophy and subsequently do not die by the same modes of death as hypertrophic chondrocytes, but rather by apoptosis (discussed in section 1.3.2). From these observations, it is clear that monolayer culture is not a suitable model to study forms of PCD of hypertrophic chondrocytes in vitro.

Several 3-D systems have been developed to maintain the differentiated state of chondrocytes such as culturing in agarose gel, alginate beads and pellet cultures (see section 1.3.1). The pellet culture method has many advantages over other 3-D culture systems. It is a simple method to obtain a cartilage-like tissue in vitro, as it involves centrifugation of cells in 15 ml tubes and keeping the tubes in an incubator. Thus it does not need any special materials. Fewer chemicals are used in pellet culture, making it cheaper than other culture systems, and allowing maintenance of a constant pH in the medium. In addition, the matrix produced by the chondrocytes in pellet culture can be identified easily by histological staining, while in other systems, staining may not differentiate between matrix synthesized by the cells and the synthetic matrix used for the culture (such as collagen or alginate). Furthermore,
pellet culture mimics native cartilage, where the cells are embedded in a matrix synthesized and degraded by the chondrocytes themselves; thus the behaviour of the cells is more similar to that of their parents in growth cartilage. For these reasons, it was decided to use the pellet culture method for the studies presented in this chapter.

The aim of the current study was to establish a 3-D system of culture in which the isolated chondrocytes from equine cartilage could be induced to undergo differentiation into hypertrophic light and dark chondrocytes and to die by the same mechanisms of PCD as those seen *in vivo.*

Horses have not previously been used as a source of chondrocytes for pellet culture, thus it was not clear what would be the optimal range of ages of animals from which cells could be harvested. In order to answer this question in the current study, chondrocytes were isolated from equine growth cartilage during different stages of development including foetal, neonatal, and older horses (growing and adult).

Because appropriate equine specimens are not available at all times, different methods of providing a continuous source of chondrocytes for culture were investigated; these included expanding the isolated chondrocytes in monolayer culture and/or freezing them in liquid nitrogen before growing them as pellets.

Foetal calf serum is used for the culture of many cell types because of its strong proliferation-inducing properties. Because the main aim of the pellet culture is to induce differentiation rather than proliferation, it was hypothesised that the horse may be a better source of serum for pellet culture of equine chondrocytes. Hence, the isolated chondrocytes were cultured in either 10% FCS or 10% HS. Furthermore, pellet culture from foetal foals was fully characterised.

### 4.2 Results

#### 4.2.1 Pellet Culture from Foetal Foals

Chondrocytes freshly isolated from growth cartilage of equine foetuses, monolayer-passaged and/or frozen chondrocytes were cultured for up to 28 days in pellets in the presence of 10% FCS, and examined by light microscopy. Cryosections were examined for alkaline phosphatase activity, and for mineralisation using the Von Kossa method. Total RNA was extracted from pellets, and expression
of the cartilage markers collagen type II and aggrecan as well as a gene typical of hypertrophic chondrocytes, collagen type X, was examined by PCR.

Nine different foetal specimens were used in the pellet culture experiments presented in this thesis. Because of the nature of the methods used, it was not possible to undertake every type of investigation for every foetus; the methods used for each specimen are indicated in Table 2.1. Light microscopic morphological studies were undertaken for pellets derived from all foetal specimens, and the qualitative observations were similar for all specimens. Figures 4.2 to 4.4 show representative examples of observations made for some of the specimens cultured in 10% FCS, as described below.

At day 0, the freshly isolated chondrocytes were rounded and closely packed together; no ECM was visible (Fig. 4.1A). Most of the cells showed the morphology of non-hypertrophic chondrocytes with healthy organelles such as RER and abundant glycogen particles (Fig. 4.1B). Some cells with nuclear condensation and cytoplasmic fragmentation typical of apoptosis (Fig. 4.1C) and a small number of cells with morphological features of necrosis such as excessive cytoplasmic vacuolation and nuclear condensation (Fig. 4.1D) were present after collagenase digestion.

With time in culture, the isolated chondrocytes formed a white disc-shaped structure with gross appearance and consistency similar to those of natural hyaline cartilage (Fig. 4.2A, Fig. 4.3A), and also expressed collagen type II and aggrecan mRNA (Fig. 4.2B, Fig. 4.3B). Histologically, the cells in culture formed a typical hyaline cartilage-like tissue formed of chondrocytes embedded in lacunae and were uniformly distributed throughout an abundant metachromatic ECM (Fig. 4.2C-F, Fig. 4.3C-E, Fig. 4.4A, B). The pellet was surrounded by a perichondrium-like layer of flattened cells. From 14 days, the pellets contained cells that appeared to be undergoing hypertrophy as confirmed by collagen type X mRNA expression (Fig. 4.2B, Fig. 4.3B), positive alkaline phosphatase staining (Fig. 4.2G, H), and increased calcification from day 14 (Fig. 4.2I, Fig. 4.4C) to day 28 (Fig. 4.2J, Fig. 4.4D).

In one experiment, the effect of 10% HS was compared with that of 10% FCS. There was no obvious difference in morphology between HS-treated pellets and FCS-treated pellets (data not shown). The growth kinetics of these pellets were
evaluated by growth parameters such as pellet diameter, weight, thickness and cellular density. The pellet diameter remained unchanged at 5 ± 1 mm after 7 days regardless of the length of time in culture and whether they were cultured in HS or FCS. The pellet weight was 5.5 ± 0.3 mg (10% FCS) and 4.5 ± 0.5 mg (10% HS) at day 7. Over 28 days in culture, the pellet weight reached 9.1 ± 0.5 mg (10% FCS) and 9.6 ± 0.6 mg (10% HS). No significant differences in pellet weight were found between the two types of serum at any time point of culture (Fig. 4.5A). The pellet thickness significantly increased over the period of culture from 143 ± 5 µm (10% FCS) and 128 ± 3.5 µm (10% HS) at day 7 to 453 ± 34 µm (10% FCS) and 445 ± 83 µm (10% HS) at day 28. No significant differences in pellet thickness were found between the two types of serum at any time point of culture (Fig. 4.5B). The cellular density was 3.8 ± 0.5 cells/mm² (10% FCS) and 2.4 ± 0.3 cells/mm² (10% HS) at day 7 and decreased to 1.2 ± 0.3 cells/mm² (10% FCS) and 1.1 ± 0.15 cells/mm² (10% HS) at day 28. The cellular density was significantly lower in 10% HS than 10% FCS at day 7, however, no significant differences in the cellular density were present between the two types of serum after 7 days (Fig. 4.5C). A similar result for increased pellet thickness and decreased cellular density was obtained with chondrocytes isolated from another foetus and cultured in 10% FCS (Fig. 4.6). In subsequent experiments with foetal cells, the focus became the later time points (21 days and later; see Chapters 5 and 6). In order to obtain enough pellets to undertake the desired experiments and perform appropriate statistical analysis, it was not possible to collect pellets at the earlier time points for analysis of growth kinetics. In all these cultures, however, it was obvious from the gross appearance that the pellets increased in thickness with time in culture.

Quantitative real time PCR was carried out to detect changes with time in expression of a number of genes known to regulate endochondral ossification, Sox9, collagen type II, aggrecan, Runx2, VEGF, CTGF, FGFR3 and MMP-13, and RP-S23 was used as a housekeeping gene. Changes in gene expression were evaluated at each time point and presented relative to expression at day 7 (Fig. 4.7).

Expression was significantly up-regulated for Sox9 (1.7-fold), collagen type II (1.8-fold) and Runx2 (2-fold) after 14 days compared with day 7, and for MMP-13 (2.8-fold) and CTGF (1.5-fold) after 21 days compared with day 7. Expression of
VEGF and FGFR3 was down-regulated from 14 days. Aggrecan mRNA expression remained unchanged. After 28 days, most of the genes examined were negatively expressed, relative to 7 days.

The morphology of chondrocytes was examined in ultrastructural studies of cells isolated from four foetuses. Similar results were obtained with all foetal isolates, and representative examples of chondrocyte morphology are shown in Fig. 4.8. The outermost layer of cells forming the perichondrium-like layer were spindle-shaped, however the cells close to this layer varied from flattened to oval in shape (Fig. 4.8A). Cells similar to those described as paralysed by Roach and Clarke (1999), were present among immature cells at day 7 and disappeared later. These cells contained a dark irregular nucleus and well developed RER and Golgi apparatus, and in some of the cells the cytoplasm contained very few organelles except for well developed Golgi apparatus (Fig. 4.8B, C). Interestingly, after 14 days, most of the cells in pellets were hypertrophic dark chondrocytes, which appeared to be dying with a similar morphology to that seen in growth cartilage (Fig. 4.8E, F). Some hypertrophic light chondrocytes, apparently dying by the same mechanism as seen in vivo, were present (Fig. 4.8G, H). In some areas, dark and light chondrocytes could be seen in the same lacunae (Fig. 4.8D). A small number of apoptotic chondrocytes were present at all stages of culture (Fig. 4.8I). After 28 days in culture, most of the cells were dying dark chondrocytes. After extensive examination of semi-thin sections and comparison with ultra-thin sections, it become possible to distinguish dark cells from light cells, and early hypertrophic from dying cells, in the semi-thin sections. Morphology of pellet cultures from 3-, 6-, 8-, and 9-month foetuses were similar.

In some experiments, chondrocytes were passaged in monolayer in the presence of 10% FCS or 10% HS before being cultured in pellets in either 10% FCS or 10% HS. In all conditions examined, the cells formed ball-like pellets, unlike the disc-shaped pellets cultured from freshly isolated cells (Fig. 4.9A) and the pellets decreased in size over time in culture. Semi-thin sections of these pellets revealed that many chondrocytes were flattened in appearance rather than rounded. The cells were similar to chondrocytes from the resting zone of growth cartilage and contained abundant glycogen (Fig. 4.9B). After 14 days in culture, many cells were necrotic.
In other experiments, the isolated chondrocytes were first frozen in liquid nitrogen for 6 months before being subjected to pellet culture in 10% FCS or 10% HS. The frozen cells formed weak and easily damaged cartilage-like tissue during the first 7-10 days of culture. The diameter of the pellets after 7 days was the same as that for the freshly isolated chondrocytes (about 5 mm). Moreover, after 14 days, histological evaluation revealed no difference in the cartilage obtained from frozen or freshly isolated cells (Fig. 4.10). No differences in growth or morphology were observed between pellets cultured in HS and those cultured in FCS.

4.2.2 Pellet Culture from Older Horses

Chondrocytes were isolated from neonatal (7-day-old), growing (6- and -12-month-old), and adult (17-year-old) horses, and cultured as pellets in the presence of 10% FCS or 10% HS. For chondrocytes isolated from neonatal foals, the pellet thickness and cellular density were measured at different time points of culture. The pellet thickness was 285 ± 57 µm (10% FCS) and 287 ± 26 µm (10% HS) at day 7, and significantly increased to reach 839 ± 96 µm (10% FCS) and 788 ± 27 µm (10% HS) at day 35 (Fig. 4.11A). The cellular density was 1.9 ± 0.05/mm² (10% FCS) and 1.8 ± 0.06/mm² (10% HS) and increased to 2.3 ± 0.17/mm² in the presence of 10% FCS, but decreased to 1.3 ± 0.15/mm² in the presence of 10% HS at day 35 (Fig. 4.11B). Pellet thickness and cellular density of pellet cultures from growing horses did not significantly change after 7 days (Fig. 4.12).

For chondrocytes obtained from neonatal, growing and adult horses and cultured in 10% FCS, the morphology of chondrocytes in pellets was similar. After 21 days in culture, pellets decreased in size, then became a ball-like structure. Histologically, the isolated chondrocytes kept their round morphology in pellets, but formed rod-like structures visible by light microscopy (Fig. 4.13A). These structures appeared as membranous inclusion-like bodies visible within the cytoplasm by electron microscopy (Fig. 4.13B, C). The ECM contained many collagen fibres similar to fibres of collagen type I (Bonucci and Motta, 1990), the main collagen in bone tissue (Fig. 4.13F). The collagen fibres were associated with mineral crystals, one of the characteristic features of osteoid mineralisation. Many cells showed an osteoblastic appearance; they had sparse organelles but dilated abundant RER, and
were embedded in an excessive amount of calcified matrix (Fig. 4.13D, E). Light and dark chondrocytes were not detectable. Some of the dying cells were necrotic and others were apoptotic.

Chondrocytes isolated from neonatal horses and cultured in 10% HS formed disc-shaped pellets similar to pellets from foetal foals. Light microscopic examination of the pellets revealed that the cells were active in secretion as evident by abundant metachromatic ECM. Furthermore the chondrocytes were undergoing terminal differentiation into dark and light cells (Fig. 4.14A). Electron microscopic examination confirmed the presence of dark (Fig. 4.14B) and light (Fig. 4.14C) chondrocytes, but the cells also contained inclusion bodies (Fig.4.14B-D).

Pellets from growing and adult horses cultured in 10% HS, were similar in appearance to those grown in 10% FCS. The number of inclusion bodies decreased, but light and dark cells were not detectable with the substitution of 10% FCS by 10% HS.

Since it was clear from these studies that pellets cultured from chondrocytes isolated from postnatal horses would be of no use for the study of chondrocyte hypertrophy and death, no further studies were undertaken with cells from postnatal horses.
Figure 4.1 Morphology of freshly isolated foetal equine chondrocytes.

Chondrocytes were isolated from a 6-month equine foetus (F6a) using collagenase digestion overnight and processed for light (A) and electron (B-D) microscopy. A: Semi-thin section stained with methylene blue. B: Normal chondrocyte; RER (R), glycogen particles (G). C: Apoptotic bodies (arrows). D: Necrotic cell; extensive vacuolation (V). Note the different patterns of nuclear condensation (N) in B-D. Bar = 25 µm in A and 1 µm (B-D).
Figure 4.2 Cartilage-like tissue formation by chondrocytes from 3-month equine foetuses.

Chondrocytes were isolated from 3-month equine foetuses (F3a, c) and cultured as pellets in 10% FCS. All parts except B are from foetus F3a; part B is from foetus F3c. Note that images of semi-thin sections of pellets from foetus F3c are shown in Fig. 6.2. A: Gross appearance of pellets cultured for 14 days. B: Agarose gel showing RP-S23 (1), collagen type II (2), aggrecan (3), collagen type X (4) and negative control (5) PCR products derived from pellets cultured for 21 days. (C-F) Semi-thin sections of pellets cultured for 14 (C), 21 (D), and 28 days (E, F). Note perichondrium-like layer (arrows) surrounding cells embedded in abundant metachromatic ECM. G, H: Cryosections stained for the presence of alkaline phosphatase activity from 21 (G) and 28 (H) day pellets; arrow indicates hypertrophic cells stained positive for alkaline phosphatase and arrowhead indicates negative reactions. I, J: Cryosections from 14 (I) and 28 day (J) pellets stained according to Von Kossa’s method; arrows indicate ECM calcification. Parts C-E, I, J have the same magnification, and parts G, H have the same magnification. Bar = 5 mm in A, 25 µm in F, 10 µm in H and 50 µm in J.
Figure 4.3 Cartilage-like tissue formation by chondrocytes from a 6-month equine foetus.

Pellets were prepared from chondrocytes isolated from a 6-month equine foetus (F6a) and cultured in 10% FCS. A: Gross appearance of 14 day-cultured pellets. B: Agarose gel showing, RP-S23 (1), collagen type II (2), aggrecan (3), collagen type X (4) and negative control (5) PCR products derived from pellets cultured for 21 days. (C-E) Semi-thin sections from pellet cultured for 14 (C), 21 (D) and 28 (E) days. Note perichondrium-like layer (arrows) surrounding chondrocytes embedded in abundant metachromatic ECM. Figure parts C-E have the same magnification; bar = 10 mm in A, and 125 µm in E.
Figure 4.4 Cartilage-like tissue formation by chondrocytes from a 9-month equine foetus.

A, B: Semi-thin sections from pellets cultured for 14 (A), and 28 (B) days in 10% FCS. C, D: Cryosections, stained according to Von Kossa’s method, of pellets cultured for 14 (C) and 28 (D) days. Arrows in C, D indicate ECM calcification. Figure parts A, B have the same magnification; bar = 125 µm. Figure parts C, D have the same magnification; bar = 25 µm.
Figure 4.5 Growth changes in pellet cultures from a 3-month foetal foal.

Chondrocytes were isolated from growth cartilage of a 3-month equine foetus (F3a) and cultured as pellets in either 10% FCS or 10% HS, and the pellets were collected after 7, 14, 21 and 28 days. The pellet weight (A), thickness (B) and cellular density (C) were measured. Data represent mean ± SEM (n ≥ 3). Significant differences are expressed as follows: a (P < 0.001), b (P < 0.01), c (P < 0.5) for comparison between each two successive time points in each serum type, f (P < 0.05) for comparison between serum types at day 7.
Figure 4.6 Growth changes in pellet cultures from a 6-month foetal foal.

Chondrocytes were isolated from growth cartilage of a 6-month equine foetus (F6a) and cultured as pellets in 10% FCS, and the pellets were collected after 7, 14, 21 and 28 days. The pellet thickness (A) and cellular density (B) were measured. Data represent mean ± SEM (n ≥ 3). Significant differences are expressed as follows: a (P < 0.001), b (P < 0.01), c (P < 0.5) for comparison between each two successive time points.
Figure 4.7 Changes in gene expression of chondrocyte pellet culture from a 6-month equine foetus.

Pellets obtained from a 6-month equine foetus (F6a) were cultured in 10% FCS for 7, 14, 21 and 28 days. Total RNA was extracted and Q-PCR was carried out to compare mRNA expression at different time points. Expression of genes of interest at days 14 - 28 normalised to RP-S23 expression is expressed relative to expression at day 7 (normalised to RP-S23 expression). Data represent mean ± SEM (n = 3).

Significant differences between values are expressed as follows: a – P < 0.001, b – P <0.01, c – P < 0.05 for comparison between each time point (14, 21 or 28 days) and 7 days.
Figure 4.8 Morphology of equine foetal chondrocytes in pellet culture.

Pellet cultures from a 3-month foetus and a 6-month foetus. Parts A, B, C, F, H are from F3b and parts D, E, G, I are from F6b. Electron micrographs from the pellets at 7 (B, C), 21 (A, I) and 28 days (D-H). A: Perichondrium-like layer, B, C: Paralysed chondrocytes. D: Proliferative light (LI) and dark (DA) chondrocytes. E, F: Hypertrophic dark chondrocytes at early (E) and late (F) stages. G, H: Hypertrophic light chondrocytes at early (G) and late (H) stages. I: Apoptotic chondrocytes. Note the pattern of nuclear condensation (N). Arrows indicate flattened cell (A), RER (B), Golgi apparatus (C), well developed RER (E), extrusion of cytoplasm into ECM (F), sparse RER (G), cytoplasmic digestion (H), and apoptotic bodies (I). Arrowhead in I indicates phagocytosis of apoptotic body by an adjacent cell. Parts B-H have the same magnification. Bars in A and I = 1 µm.
Figure 4.9 Morphology of pellet culture from monolayer-cultured chondrocytes.

Chondrocytes were isolated from a 6-month equine foetus (F6-b) and cultured in 10% FCS in monolayer before being grown as pellets for 14 days in 10% FCS, and processed for light microscopy. A: Photograph showing the difference in appearance between pellet culture from monolayer-cultured (m) and freshly isolated (f) chondrocytes. B: Semi-thin section from pellet culture of monolayer-cultured chondrocytes. Arrow indicates flattened cells and arrowhead indicates cytoplasmic glycogen. Bar = 10 mm in A and 50 μm in B.
Figure 4.10 Morphology of pellet culture from frozen chondrocytes.

Chondrocytes were isolated from a 9-month equine foetus and frozen before being grown as pellets for 21 days in 10% FCS, and processed for light microscopy and electron microscopy. A: Semi-thin section. B: Electron micrograph showing dark chondrocytes. Arrow in A indicates perichondrium-like layer surrounding chondrocytes embedded in abundant ECM, arrow in B indicates dilated RER and arrowhead in B indicates many secretory vesicles. Bar = 10 mm in A, 125 μm in B and 2.5. μm in C.
Figure 4.11 Growth changes in pellet culture from a neonatal foal.

Chondrocytes were isolated from a 7-day-old foal and cultured as pellets in 10% FCS (A) or 10% HS (B) for 35 days, and pellet thickness (A) and cellular density (B) were measured from semi-thin sections. Data represent mean ± SEM (n = 3). Significant differences are expressed as follows: a (P < 0.001), b (P < 0.01), c (P < 0.05) for comparison between successive time points in each serum type, d (P < 0.001) for comparison between the two serum types.
Figure 4.12 Growth changes in pellet culture from a growing horse.
Chondrocytes were isolated from a 6-month-postnatal horse (P6a) and cultured as pellets in 10% FCS for 28 days, and pellet thickness (A) and cellular density (B) were measured from semi-thin sections. Data represent mean ± SEM (n = 3).
**Figure 4.13 Morphology of pellet culture from a growing horse.**

Chondrocytes were isolated from growth cartilage of a 6-month-old horse (P6a), cultured for 28 days as pellets in 10% FCS and examined by light (A) and electron (B-F) microscopy.  

A: Semi-thin section stained with methylene blue.  

B, C: Membranous inclusion bodies in longitudinal (B) and transverse (C) sections.  

D, E: Osteoblast-like cells containing a large pale nucleus (N) and well developed RER (R) present in an abundant mineralised matrix (CA).  

E, F: Collagen-I like structures undergoing calcification (CA).  

Arrows indicate inclusion bodies in A, B, and C, and collagen fibrils in F.  

Arrowhead in C indicates membranes surrounding inclusion bodies.  

Bar = 25 μm in A and 1 μm in B-F.
Figure 4.14 Morphology of pellet culture from a neonatal foal.
Isolated chondrocytes were cultured for 35 days as pellets in 10% HS and processed for electron microscopy. A: Light micrograph. B-D: Electron micrographs showing hypertrophic dark (B) and light (C) chondrocyte, and intramembranous inclusion bodies (D). Arrows indicate inclusion bodies. In B, note abundant RER and well developed Golgi apparatus (G). Bar in A = 20 µm. Bar = 1 µm in figures B-D.
4.3 Discussion

The aim of the current study was to establish a system of culture in which the chondrocytes behave in a similar manner to cells in growth cartilage where the chondrocytes terminally differentiate into hypertrophic dark and light cells and then die by PCD. Chondrocytes were isolated from cartilage of horses of a variety of ages ranging from foetal to ageing adult, and cultured as pellets in the presence of either 10% FCS or 10% HS. Furthermore, foetal cells initially cultured in monolayer and/or frozen were subsequently grown as pellets. At different time points of culture, the pellets were examined by light and electron microscopy.

The morphology of the pellets revealed a considerable variability in relation to the age of the horses from which the chondrocytes were isolated. Freshly isolated chondrocytes from foetal foals formed a hyaline cartilage-like tissue as confirmed by the gross and histological appearances, and the expression of the chondrocyte markers, collagen type II and aggrecan mRNA. The chondrocytes in pellets were undergoing hypertrophy as indicated by their morphology, expression of collagen type X, alkaline phosphatase staining and increased calcification of the ECM from day 7 to day 28. Previous studies have also reported that chondrocytes cultured in pellets in 10% FCS undergo hypertrophy (Kato et al., 1988). To evaluate the growth rate of the pellet culture, pellet diameter, weight, thickness and cellular density were measured. It was found that the pellet diameter did not change after 7 days in culture. Thus, it is likely that the size of the bottom of the centrifuge tubes used in these experiments limited the pellet expansion. The pellet weight increased significantly during the period of culture. To understand whether this increase in weight was caused by ECM production or cellular proliferation, the pellet thickness and the cellular density were measured. Pellet thickness increased as cellular density decreased over time in culture, indicating that the increase in weight was due more to ECM production than cell division. It has been reported that in chondrocyte pellet culture, cell proliferation occurs mostly during the first eight days, then sharply decreases later (Yasuda et al., 1995).

Quantitative PCR studies showed that the transcription factor Sox9 along with collagen type II mRNA expression was up-regulated after 14 days, indicating
progressive differentiation over this period. It has previously been shown that Sox9 is essential for maintaining the phenotype of human cultured chondrocytes, and enhances collagen type II expression (Tew et al., 2005). The transcription factor Runx2, which stimulates hypertrophy of mouse chondrocytes (Takeda et al., 2001), was up-regulated after 14 days when hypertrophic chondrocytes were first detectable morphologically. MMP-13 and CTGF were up regulated after 21 days. CTGF has been shown to be expressed by hypertrophic chondrocytes (Fukunaga et al., 2003) and MMP-13 is essential for degradation of cartilage ECM during endochondral ossification (Inada et al., 2004). FGFR3 was down-regulated at 14 days. Activation of FGFR3 inhibits chondrocyte proliferation (Dailey et al., 2003), but also inhibits chondrocyte hypertrophy (Minina et al., 2002). Thus it is likely that down regulation of FGFR3 in pellet cultures allows cellular hypertrophy. VEGF expression was down-regulated at all time points compared to 7 days. This observation is surprising since in mice VEGF expression is up-regulated with chondrocyte hypertrophy, and dependent on Runx2 expression (Zelzer et al., 2001). It is possible that there are species differences in the regulation of VEGF expression by hypertrophic chondrocytes. Because of the amount of time required to undertake Q-PCR analyses of pellet cultures, and because such an experiment requires almost all the pellets from one isolate for RNA extraction, this experiment was only undertaken on pellets from one foetus. These results were generally in agreement with published observations for hypertrophic chondrocytes, but the experiment would need to be repeated before definitive conclusions are reached.

Pellets cultured in FCS or HS contained typical hypertrophic dark and light cells, and they appeared to be dying by the same mechanisms seen in vivo as described in chapter 3. To the best of my knowledge, this is the first study to demonstrate that isolated chondrocytes can be induced to die by the same modes of PCD as those observed in growth cartilage in vivo. In this culture system, the terminally differentiated dark and light cells mimic the last live events of their parents and die as they die.

In addition to dying dark and light cells, apoptotic cells were present during all stages of culture close to the superficial layers of the pellets. Apoptosis is routinely
described as occurring in cultured cells, including chondrocytes (Roach and Clarke, 2000).

“Paralysed” cells were also observed among immature cells in early cultures before the onset of cell hypertrophy. This type of chondrocyte was observed around the cartilage canals of some 3-month equine foetuses (L. Tatarzuch, unpublished results). Cartilage canals may be present within the digested cartilage used for culture, so it is not surprising to find such types of chondrocytes in the culture. Paralysed cells have been reported as a form of PCD present among immature chondrocytes (Roach and Clarke, 1999; Roach and Clarke, 2000). In the current work, paralysed cells had well developed RER and Golgi apparatus. In some cells the cytoplasm was completely empty except for well developed Golgi. It is not clear if the cytoplasm underwent digestion as explained by Roach and Clarke (2000) or it was filled with secretion, which dissolved during processing leaving empty spaces. Using specific staining for proteoglycans, L. Tatarzuch (un-published results) found these cells were filled with proteoglycans. Paralysed cells were no longer present after 14 days, but there was no evidence of cell debris, and the pellet growth continued after that. From these observations, paralysed cells may not be dying but they may represent a form of immature chondrocyte that performs some secretory functions during the early stages of endochondral ossification.

It was found that equine chondrocytes cultured initially in monolayer and then in pellets were similar to cells from the resting zone of growth cartilage, but failed to undergo further hypertrophic differentiation and subsequently they did not undergo typical chondrocyte PCD. Reports on the ability of monolayer-cultured chondrocytes to redifferentiate in pellets vary. It has been reported that bovine chondrocytes grown in a monolayer culture show a low capacity of redifferentiation when transferred to pellets (Malda et al., 2003). However, some investigators have reported that human chondrocytes cultured in a monolayer redifferentiate when grown in pellet culture (Oyajobi et al., 1998). Such variation is likely due to species variability (Giannoni et al., 2005). Furthermore, Oyajobi et al., (1998) reported that the redifferentiated chondrocytes express collagen type X, a gene typical of hypertrophy, but they have not shown any morphological evidence of the occurrence of hypertrophy in their system.
Frozen foetal chondrocytes cultured as pellets formed a cartilage-like tissue similar to those from freshly isolated cells after 21 days in culture, and the cells underwent hypertrophy and PCD. However, the pellets in early culture were fragile and easily broken during changes of medium. It has been reported that the viability of frozen equine neonatal chondrocytes is lower than that of chondrocytes from older horses (Nixon et al., 1992). During early culture of frozen foetal chondrocytes, the culture contained a high number of dead chondrocytes resulting in a weak cartilage, however, in later culture the pellets were more resilient and were similar to pellets from freshly isolated cells. The surviving chondrocytes may maintain differentiation and produce ECM leading to increased strength for the pellets.

Pellet cultures from growing and adult horses formed inclusion bodies. The inclusion bodies may be pro-collagen retained within cells. The ECM contained collagen with a high degree of resemblance to collagen type I, the main collagen in bone tissue, and its mineralisation was also similar to the mineralisation pattern of collagen type I. Moreover, many cells were similar to osteoblasts, and were embedded in a highly mineralised matrix. From all of these features, it seems that the cells from growing and adult horses cultured in 3-D pellets were undergoing transformation into osteoblast-like cells. Transformation of chondrocytes into bone cells has been reported in organ culture of murine cartilage (Thesingh et al., 1991), however, further studies are required to confirm this suggestion.

Pellet cultures derived from chondrocytes isolated from neonatal foals behaved in a different manner in FCS and HS. In FCS, they were similar to pellets from older horses. In HS, they contained hypertrophic dark and light chondrocytes, but also contained inclusion bodies. These different results may be due to the fact that different factors in serum can affect cell behaviour in culture.

It is likely that these differences in behaviour in culture between cells derived from animals of different ages result from differences in the tissue used for culture. In all cases, the tissue used extended from the articular surface to a point sufficiently distant from the ossification front to exclude any bone or hypertrophic chondrocytes. Thus, in all specimens it included the permanent articular cartilage. In foetal specimens, the...
majority of cells would have been resting and proliferative chondrocytes of the growth cartilage, but with increasing age, the proportion of the specimen comprised of such cells would have decreased, while the proportion comprised of permanent articular chondrocytes would have increased. The observations suggest that chondrocytes from permanent articular cartilage do not have the capacity to undergo hypertrophy in culture.

In conclusion, the present findings demonstrate that chondrocytes freshly isolated from equine foetuses and cultured as pellets undergo the same modes of dark and light cell differentiation and PCD seen in vivo. Chondrocytes from neonatal or older horses are not suitable for studying PCD in vitro. This culture system will be useful for further studies of the molecular mechanisms controlling equine hypertrophic chondrocytes and their PCD.
CHAPTER 5: MANIPULATION OF DIFFERENTIATION AND PCD IN CHONDROCYTE PELLET CULTURE

5.1 Introduction

Although many studies have used the pellet culture system to explore the effect of exogenous factors on chondrocyte morphology and ECM production (Kato et al., 1988; Ballock and Reddi, 1994; Okubo and Reddi, 2003), to date no studies have used this system to investigate differences between dark and light hypertrophic chondrocytes, or the regulation of PCD. In the previous chapter, it was demonstrated that equine foetal chondrocytes cultured in pellets undergo dark and light cell differentiation and the forms of PCD seen in hypertrophic chondrocytes in vivo. One of the aims of the studies presented in this chapter was to determine whether the proportions of dark and light cells, as well as their specific forms of PCD, could be modified by altering culture conditions. It was hoped that conditions could be found that would allow the investigation of molecular differences between light and dark, live and dying hypertrophic chondrocytes.

Altering the serum concentration in pellet culture has previously been shown to influence the rate of chondrocyte hypertrophy (Bohme et al., 1992). Transforming growth factor-β and T3 are two important antagonistic regulators of endochondral ossification; T3 stimulates chondrocyte hypertrophy (Alini et al., 1996), however, TGF-β prevents the conversion of prehypertrophic chondrocytes into hypertrophic cells (Ballock et al., 1993). For these reasons, the effects of a variety of culture conditions, including serum concentration and treatment with T3 and TGF-β1 on differentiation and PCD in chondrocyte pellet culture were investigated.

A second aim of the study presented here was to determine whether hypertrophic chondrocytes can be induced to die by apoptosis. In contrast to chondrocyte pellet culture, monolayer culture has been extensively used in chondrocyte apoptosis studies. As discussed in detail in section 1.3.2, a wide variety of factors and culture conditions can induce apoptosis in chondrocytes in monolayer culture; these include cytokines, hormones, nitric oxide and serum deprivation. One of these inducers of apoptosis,
staurosporine, is a non-selective protein kinase inhibitor and has been used widely in chondrocyte monolayer culture (Mukherjee et al., 2001; Kirsch et al., 2003; Lee et al., 2005). The ability of staurosporine to induce apoptosis in chondrocyte pellet culture was, therefore, investigated.

5.2 Results
5.2.1 Effects of Serum Type and Period of Culture on Chondrocyte Differentiation and PCD

As described in section 4.2.1, foetal chondrocytes were cultured as pellets in either 10% FCS or 10% HS for periods of up to 28 days, and examined by light microscopy. The number of dark, light, paralysed and resting cells, as well as cells undergoing different forms of PCD, were counted in 6 different locations per pellet, and expressed as a percentage of total cell number (Fig. 5.1). At day 7, most of the cells of pellets cultured in either serum type were resting, with few differentiated dark or light chondrocytes present. As mentioned in section 4.2.1, some cells similar to the cells described as paralysed chondrocytes by Roach and Clarke (2000) were present at this stage. At day 7, there were significantly more paralysed cells in 10% FCS than in 10% HS-treated pellets. Very few paralysed cells were observed in either serum type at day 14, and none were observed after this (Fig. 5.1A). At 14 days, a substantial proportion (about 40%) of cells was differentiated dark chondrocytes, and at 21 and 28 days, most of the cells were dark chondrocytes. Few light chondrocytes were present in either serum type at any time point. The proportion of resting cells decreased with time in culture, as the proportion of dark cells increased, in both serum types.

Dying dark cells were first detected at day 14, and the proportion of dying dark cells increased with time in culture (Fig. 5.1B). By day 28, more than 40% of cells in either serum type were dying dark cells. A small percentage of dying light cells was present in pellets cultured in either serum type at all time points examined. Apoptotic cells were present at all stages of culture. No significant differences in cell differentiation or PCD were noticed between serum types after 7 days in culture (Fig. 5.1B).
5.2.2 Effects of Serum Concentration, T3 and TGF-β1 on Chondrocyte Differentiation and PCD

The foetal chondrocyte pellet cultures were maintained in either 10% or 0.1% FCS in the presence or absence of T3 (100 ng/ml) or TGF-β1 (10 ng/ml) for up to 28 days (Fig. 5.2). The pellets were collected after 21 and 28 days and dark, light and resting cells, as well as cells undergoing each form of PCD were counted and expressed as a percentage of total cell number.

As shown above, in pellets cultured in 10% FCS, most cells were dark chondrocytes (about 80%) and very few light chondrocytes were present (Fig. 5.3A). In pellets cultured in 0.1% FCS, there were more light than dark chondrocytes, but a high proportion of chondrocytes remained as resting cells, not distinguishable as either light or dark cells (Fig. 5.3A). An analysis of cell death demonstrated that whereas in pellets cultured in 10% FCS, there were many dying dark cells, in pellets cultured in 0.1% FCS, the vast majority of the cells remained viable after 28 days in culture (Fig. 5.2A, B, Fig. 5.3B).

Pellets cultured with T3 in 10% FCS showed no significant differences in either dark or light cell differentiation (Fig. 5.4A) or PCD (Fig. 5.4B) from pellets grown in 10% FCS alone. In pellets cultured in the presence of T3 in 0.1% FCS, there were more light cells than dark cells. More light and dark cells were seen at 21 days and 28 days than in pellets cultured in 0.1% FCS alone (Fig. 5.5A), but at 28 days the ratio of light:dark cells was similar for the two treatment conditions (1.48 for T3-treated pellets and 1.69 for 0.1% FCS-treated pellets). Moreover, treatment with T3 led to the presence of increased percentages of dying light and dark cells by day 28 (Fig 5.5B).

Pellets grown in medium containing TGF-β1 in 10% FCS showed no significant difference from pellets grown in 10% FCS alone in relation to differentiation into dark and light cells (Fig. 5.6A). At 21 days, however, TGF-β1 caused a significant increase in the proportion of dying dark cells (Fig. 5.6B). In the presence of 0.1% FCS, TGF-β1 strongly stimulated dark cell differentiation (Fig. 5.7A). Moreover, most of the TGF-β1-induced dark cells were already dying by 21 days (Fig. 5.7B).
5.2.3 Can Hypertrophic Chondrocytes Be Induced to Die by Apoptosis?

Chondrocytes from a 3-month equine foetus (F3b) were cultured in monolayer in medium containing 10% FCS. After the cells reached confluence, 10% FCS was replaced by 0.1% FCS for 24 hours, then staurosporine (1 µM) was added for a further 24 hours. Staurosporine induced progressive changes in the culture including rounding and blebbing of the adherent cells, and shedding and floating of many cells in the medium (Fig. 5.8A, B). The adherent cells were detached by trypsin and added to the medium containing floating cells. The cells were centrifuged and the resulting pellets were processed for light and electron microscopy. The same concentration of staurosporine was added to pellets grown in 0.1% FCS at day 20. The pellets were collected after 24 hours and processed for light and electron microscopy. Histological analysis revealed that staurosporine induced apoptosis in about 43% of cells grown in monolayer culture (Fig. 5.8 C, D, Fig. 5.9A-C). In pellet culture, staurosporine did not induce apoptosis but rather induced dark cells to undergo a form of death similar to that seen in these cells in vivo (Fig. 5.8 E, F, Fig. 5.9D-F). Pellets cultured from chondrocytes isolated from a 10-month equine foetus (F10a) were also treated with staurosporine. Once again, staurosporine increased the percentage of dying dark chondrocytes (from 10% in the control pellets to 25% in the treated pellets).

5.2.4 Overview of Culture Conditions for Induction of Light and Dark Cell Differentiation and Death

The results presented in 5.2.2 suggested that 10% FCS provided the best conditions for obtaining hypertrophic dark cells and inducing their death, and that T3 in 0.1% FCS provided the best conditions for obtaining pellets containing hypertrophic light cells and inducing their death. These two culture conditions were, therefore, used with cultures of chondrocytes isolated from a number of foetal specimens. Counts of light and dark cells, as well as different types of dying cells, were conducted for each treatment for each foetal specimen. Table 5.1 summarises the specimens subjected to each of these two treatments and the figures or section numbers in which the data are
presented. All results for specimens of less than 10 months were highly consistent between specimens. The two 10-month specimens showed higher levels of cell death in 21 day pellets than specimens from younger foetuses (discussed in Chapter 6). The proportions of light and dark cells were similar to those observed for younger foetal specimens, but there was a tendency towards a higher ratio of light : dark cells in T3-treated pellets.

Table 5.1 Foetal specimens treated with culture conditions favourable for obtaining light or dark chondrocyte-enriched pellets.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>10% FCS</th>
<th>T3 in 0.1% FCS</th>
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<tbody>
<tr>
<td>F3a</td>
<td>Fig. 5.1</td>
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<tr>
<td>F3c</td>
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<tr>
<td>F6a</td>
<td>**</td>
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<tr>
<td>F6b</td>
<td>Fig. 5.3</td>
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<td>9-month</td>
<td>**</td>
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<tr>
<td>F10a</td>
<td>-</td>
<td>6.2.2, paragraph 2</td>
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<tr>
<td>F10b</td>
<td>6.2.2, paragraph 2</td>
<td>6.2.2, paragraph 2</td>
</tr>
</tbody>
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* The reference denotes the figure or section of the thesis in which cell count data are presented for different specimens.

** Data not shown elsewhere:
1- Pellet cultures from F6a contained approximately 55% dark and 8% light chondrocytes in the early stage of hypertrophy after 14 days, and 73% dark and 10% light dying hypertrophic chondrocytes after 28 days.
2- Pellet cultures from the 9-month equine foetus contained approximately 40% dark and 10% light chondrocytes in the early stage of hypertrophy after 14 days, and 84% dark and 7% light dying hypertrophic chondrocytes after 28 days.
Chondrocytes were isolated from growth cartilage of a 3-month equine foetus (F3a) and cultured in the presence of either 10% FCS or 10% HS for up to 28 days. Semi-thin sections were taken from pellets cultured for 7 days (7 D), 14 days (14 D), 21 days (21 D) and 28 days (28 D). Dark, light, resting or paralysed chondrocytes (A), and dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes (B) were counted and expressed as a percentage of total cell number. Results are presented as mean ± SEM. Significant differences between each two successive time points in either serum type are indicated as follows: a (P < 0.001), b (P < 0.01), c (P < 0.05). A significant difference between serum types at any time point is indicated as d (P < 0.001), e (P < 0.01).
Figure 5.2 Semi-thin sections from pellets cultured in 10% FCS (A), 0.1% FCS (B), T3 in 0.1% FCS (C) and TGFβ1 in 0.1% FCS (D) for 28 days.

Chondrocytes were isolated from growth cartilage of a 6-month (F6b; A-C) and a 3-month (F3b; D) foetus and cultured under a variety of conditions. A-D: Semi-thin sections stained with methylene blue. Arrows indicate chondrocytes undergoing dark cell death; arrowhead in (B) indicates a viable cell and in (C) indicates a cell undergoing light cell death. All the figure parts have the same magnification. Bar = 50 µm.
Figure 5.3 Effect of serum concentration on chondrocyte differentiation and PCD.

Chondrocytes were isolated from growth cartilage of a 6-month equine foetus (F6b) and cultured in the presence of either 10% or 0.1% FCS for 28 days. Dark, light or resting chondrocytes (A), and dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes (B) were counted after 21 days (21 D) and 28 days (28 D) and expressed as a percentage of total cell number. Data represent mean ± SEM. Significant differences between values are expressed as follows: a (P < 0.001), b (P < 0.01), c (P < 0.05) for comparison between 10% and 0.1% FCS; d (P < 0.001), e (P < 0.01) for comparison between 21D and 28D.
Figure 5.4 Effect of T3 in 10% FCS on chondrocyte differentiation and PCD.

Chondrocytes were isolated from growth cartilage of a 6-month equine foetus (F6b) and cultured in the presence or absence (control) of T3 in 10% for 28 days. Dark, light or resting chondrocytes (A), and dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes (B) were counted after 21 days (21 D) and 28 days (28 D) and expressed as a percentage of total cell number. Data represent mean ± SEM. Significant differences between values are expressed as follows: d (P < 0.001), f (P < 0.05) for comparison between 21D and 28D.
Figure 5.5 Effect of T3 in 0.1% FCS on chondrocyte differentiation and PCD.

Chondrocytes were isolated from growth cartilage of a 6-month equine foetus (F6b) and cultured in the presence or absence (control) of T3 in 0.1% FCS for 28 days. Dark, light or resting chondrocytes (A), and dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes (B) were counted after 21 days (21 D) and 28 days (28 D) and expressed as a percentage of total cell number. Data represent mean ± SEM. Significant differences between values are expressed as follows: a (P < 0.001), b (P < 0.01), c (P < 0.05) for comparison between treatments; d (P < 0.001), e (P < 0.01) for comparison between 21 D and 28 D.
Figure 5.6 Effect of TGF-β1 in 10% FCS on chondrocyte differentiation and PCD.

Chondrocytes were isolated from growth cartilage of a 6-month equine foetus (F6b) and cultured in the presence or absence (control) of TGF-β1 in 10% FCS for 28 days. Dark, light or resting chondrocytes (A), and dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes (B) were counted after 21 days (21 D) and 28 days (28 D) and expressed as a percentage of total cell number. Data represent mean ± SEM. Significant differences between values are expressed as follows: a (P < 0.001) for comparison between treatments; d (P < 0.001) for comparison between 21 D and 28 D.
Figure 5.7 Effect of TGF-β1 in 0.1% FCS on chondrocyte differentiation and PCD.

Chondrocytes were isolated from growth cartilage of a 3-month equine foetus (F3b) and cultured in the presence or absence of TGF-β1 in 0.1% FCS for 28 days. Dark, light or resting chondrocytes (A), and dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes (B) were counted after 21 days (21 D) and 28 days (28 D) and expressed as a percentage of total cell number. Data represent mean ± SEM. Significant differences between values are expressed as follows: a (P < 0.001), b (P < 0.01), for comparison between treatments; d (P < 0.001) for comparison between 21 D and 28 D.
Figure 5.8 Light micrographs from monolayer layer and pellet cultures treated with or without staurosporine.

Chondrocytes were isolated from growth cartilage of a 3-month equine foetus (F3b) and cultured as monolayer (A-D) or as pellets (E, F) in the presence (A, C, E) or absence (B, D, F) of staurosporine. A, B: Phase contrast photographs. C-F: Semi-thin sections stained with methylene blue; arrows indicate apoptotic (A, C) and dying dark cells (E). Parts A, B have the same magnification; bar = 67 μm. Parts C, D have the same magnification; bar = 25 μm. Parts E, F have the same magnification; bar = 40 μm.
Figure 5.9 Effect of staurosporine on PCD.

Chondrocytes were isolated from growth cartilage of a 3-month equine foetus (F3b) and cultured in monolayer (A-C) or as pellets (D-F). Electron micrographs of cultures treated for 24 hours in the presence (A, D) or absence (B, E) of staurosporine (1 μM). Arrows indicate the nuclear membrane, and arrowheads in (A) indicate aggregations of condensed chromatin characteristic of apoptosis. All micrographs have the same magnification; bar = 2 μm. C, F: dying dark (DDC), dying light (DLC) or apoptotic (APOP) chondrocytes were counted and expressed as a percentage of total cells. Data represent mean ± SEM. Significant differences between values are expressed as follows: a (P < 0.001 for comparison between treatment and control.
5.3 Discussion

The first aim of this study was to investigate whether chondrocyte differentiation and PCD can be modified by altering culture conditions. In experiments designed to investigate the effect of period of culture and serum type, it was demonstrated that in either 10% FCS or 10% HS at day 7, most of the cells were resting, and differentiation into dark and light cells increased with time in culture. At 21 days, the majority of the chondrocytes (more than 70%) were dark cells and only a small proportion of light chondrocytes was seen. There were no significant differences in cell differentiation between pellets cultured in FCS and HS except at day 7 when there were significantly more paralysed chondrocytes in pellets grown in 10% FCS. As discussed in section 4.3, it is not clear whether paralysed cells are dying cells as suggested by Roach and Clarke (2000). These cells constitute 50% of the total cell number in 7-day pellets grown in 10% FCS. If they were dying cells, it would be likely that at 14 days a considerable amount of cell debris would be observed in the pellets and this is not the case. Moreover, the pellets in FCS would not be expected to grow as rapidly as pellets in HS (which have almost no paralysed cells at day 7), which is also not the case (see Fig. 4.3). Thus, it seems likely that paralysed cells are not dying. For this reason they have been included in graphs showing different forms of differentiation rather than those showing forms of PCD.

Analysis of PCD in pellets grown in FCS or HS revealed that most of the dying chondrocytes at 28 days were typical dying dark chondrocytes. Few dying light or apoptotic chondrocytes were present. With respect to hypertrophy and cell death, there was no substantial difference between HS and FCS for foetal chondrocytes. Since the aim of the work was to study physiological cell death of hypertrophic chondrocytes, and since the experiment presented in Fig. 3.1 took more than 3 months (including cell isolation, culture, embedding, sectioning as well as counting of different cells in triplicate pellets for each treatment and time point), it was judged not necessary to repeat this comparison between FCS and HS, and FCS was chosen for further studies.

Further studies investigated the regulation of cell differentiation and PCD by different serum concentrations, T3 and TGF-ß1. Pellets cultured in a low serum concentration (0.1% FCS) contained a lower ratio of dark : light cells than pellets
cultured in a high serum concentration (10% FCS). Thus it is likely that the serum contains factors that favour dark differentiation of chondrocytes. Furthermore a higher proportion of resting and a lower proportion of differentiated cells were found in pellets cultured in 0.1% FCS. It is well known that serum is a potent inducer of chondrocyte hypertrophy in culture (Bruckner et al., 1989). The proportion of different forms of dying chondrocytes in pellets cultured in 0.1% FCS was significantly lower. It has been reported that chicken chondrocytes grown in a 3-D gel suspension under serum-free conditions remain viable for several weeks (Bruckner et al., 1989). Together these observations suggest that chondrocytes in 3-D culture do not need serum for their survival, but rather depend on survival signals secreted by each other, as proposed by Ishizaki, et al. (1994). In contrast, in a high serum concentration (10% FCS), a high proportions of cells were dying by 28 days suggesting that factors in serum, which has been shown to stimulate chondrocyte hypertrophy in culture (Bruckner et al., 1989), may also influence cell death.

Triiodothyronine, which initiates chondrocyte hypertrophy in cartilage explant culture (Burch and Lebovitz, 1982; Burch and Van Wyk, 1987), in femur organ culture (Wakita et al., 1998), in agarose suspension culture (Robson et al., 2000) and in pellet culture (Okubo and Reddi, 2003), was found to induce differentiation and death in chondrocyte pellets cultured in a low serum concentration (0.1% FCS), but did not appear to selectively induce differentiation of either type of chondrocyte (light or dark). It is likely that T3 stimulates the latest stages of terminal differentiation: hypertrophy and death. T3 in a high serum concentration had no effect on cell death. It is likely that cells are maximally responsive to 10% FCS making no further response to T3 possible.

Transforming growth factor-ß1, which has been shown to maintain chondrocytes in a prehypertrophic state (Ballock et al., 1993), increased the proportion of dark chondrocytes in pellets grown in 0.1% FCS but had no effect on pellets cultured in 10% FCS and accelerated dark, but not, light cell death in chondrocyte pellet culture. This result is in agreement with many previous reports suggesting that TGF-ß regulates cell death in a variety of cells including chondrocytes. It has been reported that TGF-ß release and activation is associated with death of chicken hypertrophic chondrocytes (Gibson et al., 2001).
Neutralization of TGF-β results in a decrease in the death of chicken embryo retinal cells (Dunker et al., 2001) and neuronal cells (Krieglstein et al., 2000). Furthermore, cell death significantly decreases in the interdigital spaces of developing limbs of TGF-β knockout mice (Gibson et al., 2001). The fact that TGF-β1 induced death of dark cells that had not undergone full hypertrophy is interesting, because it suggests that the form of death undergone by dark cells is independent of the process of hypertrophy, at least beyond a certain stage of differentiation.

The most important aim of the studies presented in this chapter was to determine whether it was possible to manipulate light and dark cell differentiation and death in pellet culture, so that the culture system could be used in the future to characterise molecular differences between the cell types. It was determined that 10% FCS provided optimal conditions for obtaining a high proportion of dark cells and inducing their death, and T3 in 0.1% FCS provided the best conditions for obtaining light cells and inducing their death. These conditions were, therefore, chosen for further studies characterising differences between dark and light cells (presented in Chapter 6). As presented in Table 5.1, results for cell proportions for 10% FCS were obtained from 7 horses. These values were extremely consistent for the 6 foetuses aged between 3 and 8 months. Results for T3 in 0.1% FCS were obtained from 5 foetuses and the results for pellets obtained from the 3 foetuses aged between 3 and 8 months were consistent. Where an initial experiment offered no advantage over another previously characterised treatment, the experiment was not repeated. Because TGF-β treatment did not provide any apparent advantage over 10% FCS in inducing dark hypertrophic chondrocyte differentiation and death, experiments using TGF-β were not repeated. These experiments would need to be repeated to reach definitive conclusions about the effects of TGF-β in the pellet culture system.

Another aim of this study was to investigate whether hypertrophic chondrocytes can be induced to die by apoptosis. Staurosporine was chosen because it is known as a potent inducer of apoptosis in a variety of cell types cultured in monolayer, including chondrocytes (Mukherjee et al., 2001; Kirsch et al., 2003; Lee et al., 2005). In the current experiment, staurosporine induced apoptosis in equine foetal chondrocytes grown in monolayer culture. The same concentration of
staurosporine was added to the chondrocytes grown in pellets with the aim of inducing apoptosis in hypertrophic chondrocytes, so that the mechanism of apoptosis could be compared with those of the forms of PCD seen in vivo. Surprisingly, staurosporine did not induce apoptosis in hypertrophic chondrocytes in pellet culture, but induced dark cell death similar to the mode of cell death seen in vivo. This result suggests that the mode of death undergone by chondrocytes is determined by their 3-D environment and/or state of differentiation rather than by the nature of the death stimulus. In addition, the possibility that there are common pathways for apoptosis and dark cell death cannot be excluded.

A number of conclusions can be reached from the results presented here. First, the ratio of dark : light chondrocytes can be modulated in pellet culture by altering culture conditions. A high serum concentration favours dark chondrocyte differentiation, whereas a low serum concentration favours (slightly) light chondrocyte differentiation. Second, the rate of chondrocyte death can be modulated in pellet culture. Triiodothyronine induces death of both light and dark cells, and a high serum concentration induces death of dark cells. Third, hypertrophic chondrocytes do not appear to be capable of undergoing apoptosis. Finally the results in this chapter support the suggestion made in Chapter 3 that light and dark chondrocytes represent two different populations of cells that undergo different non-apoptotic modes of death. If dark and light cells were different stages of differentiation of one cell type, it would be expected that with time in culture the ratio of light : dark cells would be reversed, but that was not seen with any of the treatments used. Moreover, cells similar to those identified in Chapter 3 as dying light chondrocytes and dying dark chondrocytes appeared at day 28 of culture in similar proportions to the early hypertrophic light and dark (respectively) chondrocytes seen at day 21, for any particular treatment. Thus, the results presented here are consistent with the conclusion that light and dark hypertrophic chondrocytes are two post-proliferative populations that die by different non-apoptotic modes of death.
CHAPTER 6: FURTHER CHARACTERISATION OF DYING DARK AND LIGHT CHONDROCYTES IN PELLET CULTURE

6.1 Introduction

In the previous chapters, *in vivo* and *in vitro* observations suggested that following proliferation, the chondrocytes in growth cartilage differentiate into two morphologically different cell populations, dark and light cells. Furthermore, dark and light chondrocytes appeared to die by different, cell-type specific non-apoptotic processes. However, other than ultrastructural differences, nothing is known about the difference between these putative cell populations. Therefore the aim of the current study was to investigate whether there are molecular differences between hypertrophic light and dark chondrocytes.

The approach taken was to make use of methods developed in studies described in Chapters 4 and 5 to obtain pellets enriched in early or dying light cells or early or dying dark cells. The pellets were used for RNA extraction and Q-PCR analysis of expression of a variety of genes known to regulate endochondral ossification; Sox9, collagen types I and II, Runx2, aggrecan, VEGF, FGFR3, CTGF and MMP-13. Runx 2 (Yoshida *et al.*, 2004) and CTGF (Fukunaga *et al.*, 2003) stimulate chondrocyte hypertrophy. Sox9 stimulates chondrocyte proliferation but inhibits chondrocyte hypertrophy (Akiyama *et al.*, 2002) and FGFR-3 activation by FGF-18 (Ornitz and Marie, 2002) inhibits chondrocyte proliferation (Dailey *et al.*, 2003) and hypertrophy (Minina *et al.*, 2002). VEGF stimulates angiogenesis (Gerber *et al.*, 1999). Runx2 (Zelzer *et al.*, 2001) and CTGF (Ivkovic *et al.*, 2003) up-regulate VEGF. MMP-13 is important for degradation of cartilage ECM (Wu *et al.*, 2002). Figure 6.1 summarises the role of these factors in the process of endochondral ossification.

Cathepsin B expression was investigated using immunohistochemistry in pellets enriched with dying light or dark chondrocytes. Cathepsins are a group of intracellular lysosomal proteolytic enzymes, which play an important role in degradation of collagen and proteoglycans (Poole and Mort, 1981). Cathepsin B has
been known for many years to be expressed by chondrocytes (Ali, 1967). It has been shown that cathepsin B is involved in apoptosis of T cells (Michallet et al., 2003).

Figure 6.1 Regulation of endochondral ossification by different factors used in the Q-PCR studies.

A-Proliferative, B-Prehypertrophic, C-Hypertrophic chondrocytes, D-ECM degradation, E-Blood vessel invasion of cartilage. Arrows indicate stimulation, crossed lines indicate inhibition, and scissors indicates proteolytic cleavage.
6.2 Results

6.2.1 Differences in Gene Expression between Hypertrophic Dark and Light Chondrocytes

Chondrocytes were isolated from the growth cartilage of equine foetuses aged 3 and 8 months. Some chondrocytes were cultured as pellets in the presence of T3 in 0.1% FCS to obtain pellets enriched with light cells and to induce their death. Some chondrocytes were cultured in 10% FCS to induce dark cell differentiation and death. The cultures were collected after 21 days (3-month foetus F3c) and 25 days (8-month foetus), and some pellets were processed for histological examination to ensure that they contained the expected proportions of early and dying hypertrophic light and dark cells. RNA was extracted from 30 pellets (three pools) per treatment at each time and used for Q-PCR to study the expression of MMP-13, CTGF, VEGF, Runx2, FGFR3, aggrecan, collagen type II and collagen type I. RP-S23 was used as a housekeeping gene.

As expected from the results shown in Chapter 5, the histological examination of the pellets revealed that pellets cultured in T3 in 0.1% FCS contained a high proportion of early (after 21 days) and dying (after 25 days) hypertrophic light chondrocytes (Fig. 6.2A, C). At 21 days, approximately 49% of the cells were light and 32% of the cells were dark chondrocytes in an early stage of hypertrophy. After 25 days, the cultures contained approximately 53% light and 20% dark dying hypertrophic chondrocytes. Pellet cultures in 10% FCS contained a high proportion of early (after 21 days) and dying (after 25 days) hypertrophic dark chondrocytes (Fig. 6.2B, D). At 21 days, they had approximately 72% dark and 8% light chondrocytes in the early stage of hypertrophy. After 25 days, the cultures contained approximately 67% dark and 25% light dying hypertrophic chondrocytes.

Quantitative PCR was carried out to compare gene expression of pellets cultured in T3 (light cell-enriched cultures) and 10% FCS (dark cell-enriched cultures) after 21 and 25 days. The Q-PCR analysis revealed significant differences in mRNA expression of some genes after 21 days. MMP-13, VEGF, CTGF, collagen type I and Sox9 were significantly more expressed in dark cell-enriched than in light cell-enriched cultures (Fig. 6.3A). The highest level of relative expression was shown by MMP-13, which was 14-fold more highly expressed in
dark cell-enriched cultures than light cell-enriched cultures, however, collagen type II was 7-fold more highly expressed in light cell-enriched cultures than in dark cell-enriched cultures.

After 25 days, the difference in CTGF (15-fold), VEGF (8-fold), MMP-13 (25-fold) and collagen type I (10-fold) expression between dying dark cell-enriched cultures and dying light cell-enriched cultures was greater than for the early hypertrophic cultures, but Sox9 was no longer differentially expressed (Fig. 6.3B). Type II collagen and Runx2 were more highly expressed in dying light cell-enriched cultures than in dying dark cell-enriched cultures (Fig. 6.3B). Aggrecan and FGFR3 were not significantly differently expressed between dark and light cell-enriched cultures at 21 or 25 days.

6.2.2 Histochemical Studies of Dying Hypertrophic Dark and Light Chondrocytes

For histochemical studies of dark cell-enriched and light cell-enriched or resting cell-enriched pellets, cells isolated from a 10-month foetus (F10b) were cultured for 21 days in 10% FCS, T3 in 0.1% FCS or 0.1% FCS alone, respectively and embedded in either Spurr’s resin or OCT compound. Semi-thin sections were stained with methylene blue, and cryosections were stained with haematoxylin and eosin.

As noted in section 5.2.4, examination of the semi-thin sections revealed a higher proportion of dying cells in 10% FCS- or T3-treated cultures than seen in 21-day pellets from younger foetuses. In pellets cultured in 10% FCS, a high proportion (95%) of chondrocytes were dying dark cells (Fig. 6.4A); with T3 a high proportion (70%) of chondrocytes were dying light cells (Fig. 6.4B) and in pellets grown in 0.1% FCS, a high proportion (75%) of chondrocytes were resting cells (Fig. 6.4C). It is likely that the increased rate of cell death in this experiment was due to the age of the foetus (10 months), since pellets from another 10-month foetus (F10a) showed similarly high numbers of dying cells; in T3 in 0.1% FCS at 21 days, 90% of chondrocytes from F10a were dying light cells.

Cryosections from pellets treated under different conditions were stained with haematoxylin and eosin. The cytoplasm of the cells in different treatments was
differentially stained with eosin. Resting chondrocytes had cytoplasm with a foamy appearance (Fig. 6.4D), light chondrocytes had well stained acidophilic cytoplasm (Fig. 6.4E) and dark chondrocytes had very little cytoplasm surrounding the nucleus (Fig. 6.4F).

For optimisation of immunohistochemistry using cathepsin-B antibody, different concentrations of primary and secondary antibody as well as different incubation times were used on cryosections of tissue specimens from equine growth cartilage. The distribution of cathepsin B in these sections was consistent with previously published observations, therefore the results are not presented here. Cryosections of pellets (from a 3-month foetus; F3a) cultured in 10% FCS were prepared. Cathepsin B antibody was used on sections from 14-day pellets (when the cells were mostly early dark hypertrophic chondrocytes) and from 28 days (when the cells were mostly dying dark hypertrophic chondrocytes). Most of the cells at both time points showed specific staining with the cathepsin B antibody, while the parallel antibody control sections appeared almost completely blank. No differences in staining between sections from the two time points were observed (Fig. 6.5). Cathepsin B immunostaining was carried out on cryosections of pellets from a 10-month foetus (F10b) treated for 21 days in 0.1% FCS, T3 in 0.1% FCS or 10% FCS. Most of the cells in all treatments expressed cathepsin B with no obvious difference between treatments (Fig. 6.6). Since these experiments provided no evidence of differences in cathepsin B localisation between early and dying hypertrophic chondrocytes, or between light and dark hypertrophic chondrocytes, no further experiments were conducted with cathepsin B immunohistochemistry.
Figure 6.2: Induction of different cell types in pellet culture.

Chondrocytes were isolated from the growth cartilage of equine foetuses aged 3 months (F3c; A, B) or 8 months (C, D) and cultured as pellets in the presence of T3 (A, C) or 10% FCS (B, D) for 21 days (A, B) or 25 days (C, D). Arrows indicate early hypertrophic light (A) and dark (B), and dying hypertrophic light (C) and dark (D) chondrocytes. Parts A-D have the same magnification; bar = 25 μm.
Fig. 6.3 Quantitative real time PCR analysis of mRNA expression in early (A) and dying (B) hypertrophic chondrocytes in culture

Pellets were cultured with chondrocytes isolated from a 3-month foetus (A; F3c) and an 8-month foetus (B). Cultures were enriched for dark and light cells by treatment with 10% FCS or T3, respectively, until day 21 (A) or day 25 (B). Results for Sox9, aggrecan, CTGF, VEGF, MMP-13, FGFR3 and collagen type I (Coll-I) expression in dark cell-enriched cultures are expressed relative to expression in light cell-enriched cultures. Results for type II collagen (Coll-II) and Runx2 expression in light cell-enriched cultures are expressed relative to expression in dark cell-enriched cultures. Data represent mean ± SEM; n = 3. Significant differences between values for light and dark chondrocytes are expressed as follows: a – P < 0.001; b – P < 0.01.
Figure 6.4 Light micrographs of pellets cultured in different conditions.

Chondrocytes were isolated from a 10-month equine foetus (F10b) and cultured as pellets in 0.1% FCS (A, D), T3 in 0.1% FCS (B, E) or 10% FCS (C, F). A-C: Semi-thin sections stained with methylene blue; arrows indicate viable resting (A), dying light (B) and dying dark (C) chondrocytes. D-F: Cryosections stained with haematoxylin and eosin; arrows indicate foamy (D), acidophilic (E) or sparse (F) cytoplasm. Parts A-C have the same magnification; bar = 125 μm. Parts D-F have the same magnification; bar = 40 μm.
Fig. 6.5 Cathepsin B immunostaining in pellet culture from a 3-month equine foetus.

Chondrocytes isolated from a 3-month equine foetus (F3a) were cultured as pellets in 10% FCS for 14 (A, B) or 28 (C, D) days. Cryosections of pellets were incubated with anti-human cathepsin B (A, C) or normal sheep serum (B, D). Parts A-D have the same magnification, bar = 125 μm.
Figure 6.6 Cathepsin B immunostaining in pellet culture from a 10-month equine foetus.

Pellets were obtained from F10b and cultured in 0.1% FCS (A, D), T3 in 0.1% FCS (B, E) or 10% FCS (C, F) for 21 days. Cryosections were incubated with anti-human cathepsin B (A-C) or normal sheep serum (D-F). Parts A-F have the same magnification; bar = 125 μm.
6.3 Discussion

The Q-PCR studies presented here suggest that there are differences in gene expression between dark and light hypertrophic chondrocytes. Hypertrophic dark chondrocyte-enriched pellets expressed significantly higher levels of Sox9, CTGF, MMP-13, VEGF and collagen type I than did hypertrophic light chondrocyte-enriched pellets. However, hypertrophic light chondrocyte-enriched pellets expressed a significantly higher level of collagen type II mRNA than did hypertrophic dark chondrocyte-enriched pellets.

CTGF, VEGF (Ivkovic et al., 2003) and MMP-13 (Alvarez et al., 2005) are expressed by hypertrophic chondrocytes, and all these genes have been reported to regulate the last stages of endochondral ossification, including ECM degradation (Inada et al., 2004) and invasion of the cartilage matrix with blood vessels (Gerber et al., 1999). As expression levels of these genes are higher in hypertrophic dark chondrocyte-enriched pellets than in hypertrophic light chondrocyte-enriched pellets, dark chondrocytes located close to the bone surface in growth cartilage in vivo may play more important roles in ECM degradation and cellular invasion. In CTGF-deficient mice, VEGF expression decreases indicating the role of CTGF as a key regulator of angiogenesis of growth cartilage (Ivkovic et al., 2003). Moreover, VEGF acts as a chemoattractant factor for osteoclasts (Gerber et al., 1999). It has been reported that CTGF also plays an important role in regulation of osteoblastic expression of MMP-9, which is needed for further remodeling of calcified cartilage matrix (Ivkovic et al., 2003) and this effect may be through up-regulation of VEGF (van der Eerden et al., 2003). Collagen type I was significantly more expressed by dark cell-enriched cultures than light cell-enriched cultures. It has been reported that collagen type I is expressed by the flattened cells of the perichondrium-like layers surrounding the pellets (Zhang et al., 2004), and that pellets cultured in 10% FCS have thicker perichondrium-like layers than pellets grown with T3 (Ballock and Reddi, 1994). Therefore, the higher expression of collagen type I in dark cell-enriched cultures may be related to the thicker flattened cell layers of the perichondrium and may not be due to the higher proportion of hypertrophic dark chondrocytes in the cultures.
After 25 days, most of the cells in both types of culture were dying either as dark (in 10% FCS) or light (in T3) hypertrophic cells. The Q-PCR results were similar to the results from the 21-day cultures, despite differences in ages of the two animals from which the cells were initially isolated. Because the two time points were conducted on different specimens, no direct comparison between early and dying hypertrophic chondrocytes can be made, but at 25 days, Sox9 was no longer more highly expressed in dark cells than light cells, and Runx2 was more highly expressed in light cells than dark cells. The experiments would need to be repeated for each time point to reach definitive conclusions about specific differences between dark and light cells at each stage of differentiation. Nevertheless, the consistency of the results between experiments conducted on cells from the two foetuses strongly suggests that there are indeed molecular differences between light and dark cells, further supporting the conclusion that these are two different populations of hypertrophic chondrocytes. The consistency of the results also indicates that Q-PCR of cultured pellets is an appropriate method for examining differences between light and dark hypertrophic chondrocytes. The only limitation of this method is the time taken to get sufficient material to undertake statistical analyses. The two experiments presented in Fig. 6.3 required at least 30 pellets per treatment, thus it was only possible to investigate one time point with the material from one foetus. The two experiments collectively took 3 months to conduct.

In the experiments designed to provide cryosections of pellets for histochemical studies, it was noted that the proportion of dying cells was greater in 10% FCS and T3 treatment than in the studies presented earlier in this thesis. There were two experiments conducted on cells from foetuses aged 10 months, and for both foetuses higher rates of death were observed in 21-day pellets than seen in 21-day pellets from younger foetuses so it is likely that the difference was due to the age of the foetus from which the cells were derived.

Cathepsin B immunostaining of cryosections could not differentiate between different cell types as most of the cells in all treatments and time points expressed cathepsin B. The presence of cathepsin B in chondrocytes has been reported in many studies. Early studies reported the presence of cathepsin B in both proliferative and hypertrophic zones (Ali, 1967). However, cathepsin B is more active in superficial
layers than deep layers of the articular cartilage (Bayliss and Ali, 1978). It was reported that cathepsin B is expressed by chondrocytes in the resting and hypertrophic zones of equine articular cartilage (Glaser et al., 2003) as well as undifferentiated equine chondrocytes grown as a monolayer (Hernandez-Vidal et al., 1998). The results presented in this study show that cathepsin B is expressed by most cells in pellet cultures, however, this study did not identify to what extent this cathepsin B is enzymatically active in different cell types. Thus, it is not clear from this study whether cathepsin B is involved in dark or light cell death.

In conclusion, the current study demonstrates that this system of culture can be used to analyse the differences in gene expression between dark and light hypertrophic chondrocytes, and suggests that dark and light hypertrophic chondrocytes are different populations of cells not only morphologically but also at the molecular level.
CHAPTER 7: GENERAL DISCUSSION

The current study has been conducted to understand how the hypertrophic chondrocytes in equine growth cartilage die. Electron microscopy of samples from AECG and PGC of the head of the humerus in foetal and postnatal horses revealed that two different types of chondrocytes were consistently present in the late proliferative and hypertrophic zones, dark and light cells. A careful qualitative and quantitative study of the morphology of light and dark cells in the different zones of growth cartilage suggested that these two cell types constituted two populations, each undergoing distinctive morphological changes with increasing proximity to the ossification front.

The fact that the proportion of light and dark cells in pellets could be manipulated by altering culture conditions supports the conclusion that light and dark cells are two different populations. The fact that with time in culture light and dark cells underwent the same morphological changes observed for these cells with proximity to the ossification front in vivo, also support the conclusion that they are two populations. Moreover, the fact that patterns of gene expression varied between light and dark cell-enriched cultures provides further evidence that these cells are two different populations.

Dark chondrocytes had well developed RER and Golgi apparatus with many secretory vesicles, however light cells had less developed RER and Golgi apparatus. It is not clear if dark and light cells are morphologically diverse because they have different pathways of secretion or because the nature of their secretion is different. The quantitative analysis of light and dark cells in tissue specimens indicated that there are relatively more light cells in the AEGC than in the PGC, as previously observed in pigs (Carlson et al., 1985). It is likely that the two cells types serve different functions, and that their proportions are adapted for the needs of the specific anatomical location.

These cells were observed to die by mechanisms that are morphologically different from other previously described modes of PCD such as apoptosis and autophagic cell death, as well as from necrosis (pathological cell death); a comparison between observations reported in the current study for dying light and dark chondrocytes and published observations on apoptosis, autophagic cell death and necrosis is shown in table 8.1.
Table 8.1 Summary of morphological characteristics of different forms of cell death.

*Part A refers to previously published works and Part B refers to observations reported in this thesis.

<table>
<thead>
<tr>
<th>Features</th>
<th>PART A* (Wyllie et al., 1980; Bursch, 2001)</th>
<th>PART B*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apoptosis</td>
<td>Autophagic cell death</td>
</tr>
<tr>
<td>Nuclear chromatin</td>
<td>Condensed into sharply circumscribed round or crescent masses at the margin of nuclear membrane</td>
<td>Condensed into single pyknotic mass in centre of nucleus</td>
</tr>
<tr>
<td>Nuclear membrane</td>
<td>Progressively convoluted; initially well preserved, later discontinued</td>
<td>Well preserved</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Condensed; well preserved organelles compacted together</td>
<td>Contains numerous autophagocytic vacuoles</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>Well preserved; often convoluted</td>
<td>Well preserved</td>
</tr>
<tr>
<td>Final stage</td>
<td>Nucleus and cytoplasm fragmented into membrane - bound apoptotic bodies</td>
<td>Organelles degraded (amorphous cytoplasm); compacted nucleus</td>
</tr>
<tr>
<td>Timing of nuclear and cytoplasmic changes</td>
<td>Nuclear changes precede cytoplasmic alterations</td>
<td>Cytoplasmic changes precede nuclear alteration</td>
</tr>
<tr>
<td>Removal of cellular remnants</td>
<td>Early phagocytosis</td>
<td>Late phagocytosis</td>
</tr>
</tbody>
</table>

N.B Part A was presented as table 1.2 on page 29.
The fact that chondrocytes do not die by apoptosis has been discussed in detail by Roach and Clarke (2000). However, these authors described the death of dark chondrocytes, but did not explain the death of the other cells in the same sections (light cells). The current study was conducted on foetal (3-10-month postconception) and immature (6-month postnatal) horses, however it is not clear how chondrocytes in articular cartilage from adult animals die. It has been reported that chondrocytes in articular cartilage from 37-72 year-old humans with rheumatoid arthritis die by apoptosis (Kim and Song, 1999). Huser et al (2006) have reported the occurrence of apoptosis in their model of osteoarthritis using cartilage explants from articular cartilage of adult horses (2-22 years). However, the images they have shown as apoptosis are similar to those presented in this study as dying dark cells.

The term chondroptosis has recently been introduced to describe dark cell death in rabbit growth cartilage (Roach et al., 2004). This term is not very appropriate because it implies that dark cell death is a form of apoptosis and that it is the only form of chondrocyte death, however the current study suggests that light and dark chondrocytes die by two different non-apoptotic mechanisms. The suggestion that hypertrophic chondrocytes may die by autophagic cell death (Shapiro et al., 2005) was ruled out by the electron microscopic observations of the absence of the increased number of lysosomal bodies typical of this form of death.

The form of death undergone by dark chondrocytes appeared to involve disintegration by breaking off packets of cytoplasmic contents and plasma membrane. Light chondrocytes, in contrast, appeared to disintegrate by digestion of their cytoplasmic contents within the cell membrane. RER was visible during all stages of cell degeneration in both cell types, indicating that dark and light cells are both active in protein synthesis and probably ECM secretion until the last stages. The Q-PCR results presented in chapter 6 suggest that death of dark and light hypertrophic chondrocytes is associated with active secretion of materials important for remodelling the surrounding cartilage ECM such as MMP-13 (Inada et al., 2004) and for osteogenic invasion such as VEGF (Gerber et al., 1999). The results suggest that dark cells express significantly higher levels of MMP-13, VEGF, CTGF and Sox9 than light chondrocytes, and that light cells express significantly higher levels of collagen type II (and Runx2) than dark cells. This different pattern of gene expression suggests that it is possible that both cell populations coordinate during the
course of their degeneration to regulate the biological changes in growth cartilage that contribute to endochondral ossification.

Among the different factors, 10% FCS was found to result in cultures with almost pure dark chondrocytes, whereas treatment with T3 (100 ng/ml) in 0.1% FCS was reported as the ideal condition for producing light cell-enriched cultures. Transforming growth factor-ß, in contrast, appeared to preferentially induce the early stages of dark cell differentiation, and to induce them to die prematurely, but in a manner morphologically similar to that undergone by more mature dark cells. These observations will be very useful in designing future studies on hypertrophic chondrocyte differentiation and death.

Figure 7.1 presents a model for the fate of chondrocytes in growth cartilage, taking into account the observations made in the in vivo studies and in the in vitro studies using specimens from foetal horses. According to the model, following proliferation, chondrocytes differentiate into either light or dark cells. The proportion of chondrocytes that differentiate into light rather than dark cells can be influenced by soluble environmental factors, as can the progression to hypertrophy and subsequently death.

The current study focussed on normal growth cartilage from immature animals; in the future studies it would be interesting to investigate the existence of light and dark cells in adult normal and diseased articular cartilage. Future work may include using this culture system not only to examine differences in gene expression but also for functional studies such as use of caspase and other protease inhibitors, signalling pathway inhibitors and gene manipulation experiments. Also this culture system could be used to investigate additional roles of growth factors and hormones in PCD of hypertrophic chondrocytes. Moreover, it may be possible to modify this system so that it could be used as a model for studies on osteochondrosis or osteoarthritis; such studies could lead to improved therapeutic agents for these diseases.

In summary, the current study provides compelling evidence that light and dark hypertrophic chondrocytes comprise two different populations that die by mechanisms distinct from apoptosis. A system of culture has been developed for induction of dark and light cells in vitro. The study showed the possibility of manipulating the proportion of each cell type as well as dying cells by altering culture conditions, and suggested that light and dark cells are different in their patterns of gene expression.
Figure 7.1 Model for the fate of chondrocytes in growth cartilage.

A: Resting chondrocytes. B, C: Proliferative light (B) and dark (C) chondrocytes. C-E: Hypertrophic light chondrocytes at early (C), middle (D) and late (E) stages. G-I: Hypertrophic dark chondrocytes at early (G), middle (H) and late (I) stages.


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