n-3 Polyunsaturated Fatty Acid Effects on
Inflammatory Mediator Activity and Intracellular
Signalling Pathways in Chondrocyte Metabolism

Samantha Hurst, BSc (Hons)

PhD Thesis 2004

Thesis submitted in fulfilment of the requirements of the degree
of Doctor of Philosophy, University of Wales

Connective Tissue Biology Laboratories
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Date .............................................
ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor Bruce Caterson and Professor John Harwood, for their input and guidance throughout the duration of my course. I would like to give a special mention to Dr. Clare Curtis for her continuous help and encouragement and for answering my endless questions! A big thanks also goes to all the other members of CTBL, especially Lab E1.03 for being great colleagues and friends.

I especially wish to thank my partner, Andy for the love, patience and support he has shown me over the last few years. Lastly, I want to thank my parents, brother and sister, and I must not forget my one year old nephew, Alfie for always making me laugh, albeit unintentionally.
ABSTRACT

Previous studies have shown that supplementation of n-3 polyunsaturated fatty acids (PUFAs) has a beneficial effect on reducing the expression and activity of degradative enzymes and inflammatory factors known to cause damage and destruction of cartilage in arthritic diseases. However, at present little is known about the intracellular signalling mechanisms involved in n-3 PUFA abrogation of these pathological manifestations. The aims of this thesis was to use a well-established in vitro model of cartilage degradation to further these studies and to investigate how n-3 PUFAs effect the expression of inflammatory factors at a proteomic level and to use specific inhibitors to identify possible signalling pathways involved in cartilage metabolism.

The results of this thesis research indicate that n-3 PUFAs abrogate IL-1-induced cyclooxygenase-2 (COX-2) mRNA expression, protein levels and activity, measured as PGE$_2$ production, in both normal bovine and human osteoarthritic articular cartilage chondrocytes. These studies were followed by the use of a simple array system to analyse the expression of several marker genes from different signalling pathways after IL-1 exposure, plus or minus n-3 PUFA supplementation. This led us to identify three possible pathways involved in IL-1-induced cartilage catabolism and inflammation. These were analysed further with the use of specific inhibitors to ascertain whether the inhibition profiles were similar to those seen by n-3 PUFAs. Two main pathways, the extracellular signal-regulated kinase (ERK) pathway and NFkB pathway were identified. Further analysis using the ERK pathway inhibitor, U0126, showed that it decreased IL-1-induced glycosaminoglycan release from the tissue, endogenous aggregcanase activity, ADAMTS-4 (but not ADAMTS-5) mRNA levels, MMP-3 and MMP-13 mRNA levels, COX-2 message, protein levels and PGE$_2$ production in a manner similar to that seen with n-3 PUFA supplementation. Collectively, these results suggest that n-3 PUFAs may be directing their effects through the ERK pathway.
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ABBREVIATIONS

aa    amino acids
AA    arachidonic acid
ADAM  A Disintegrin and Metalloproteinase Domain
ADAMTS A Disintegrin and Metalloproteinase with ThromboSpondin motifs
ANOVA analysis of variance
AP-1  activating protein-1
AP-2  activating protein-2
ATF   activating transcription factor
BCIP  5-bromo-4-chloro-1-indolyl phosphate
Bcl-3  B-cell leukaemia oncogene-3
B_o   maximum binding
BSA   bovine serum albumin
C/EBP CCAAT/enhancer binding protein
Ca^{2+} Calcium ions
cAMP  cyclic adenosine monophosphate
CDK   cyclin dependant kinases
cDNA  complementary DNA
CHOP  C/EBP homologous transcription factor (or Gadd53 - growth arrest and DNA damage gene 153)
CILP  cartilage intermediate layer protein
CKI   cyclin dependant kinase inhibitors
COL   collagenous domain
COMP  cartilage oligomeric protein
COX   cyclooxygenase
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<td>COX-2</td>
<td>cyclooxygenase-2</td>
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<td>CRE</td>
<td>cyclic AMP responsive element</td>
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<tr>
<td>CRP</td>
<td>complement regulatory protein</td>
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<td>Elk-1</td>
<td>a ternary complex factor transcription factor containing an Ets domain</td>
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LOX  lipoxygenase
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12-LOX  12-lipoxygenase
15-LOX  15-lipoxygenase
LP1  link protein 1
LP2  link protein 2
LP3  link protein 3
LPS  lipopolysaccharide
LT  leukotrienes
LTB₄  leukotrine B₄
MAb  monoclonal antibody
MAPEG  membrane-associated proteins in eicosanoids and glutathione metabolism
MAPK  mitogen activated protein kinase
MAPKK  mitogen activated protein kinase kinase (MAPK kinase)
MAPKKK  mitogen activated protein kinase kinase kinase (MAPKK kinase)
MEK  ERK kinase
MEKK  ERK kinase kinase (MEK kinase)
MHC  major histocompatibility complex
MMLV  Moloney murine leukaemia virus
MMP  matrix metalloproteinase
MSF  megakaryocyte stimulating factor
MuLV  murine leukaemia virus
MyD88  myeloid differentiation primary response gene 88
NBT  nitroblue tetrazolium
NC  non-collagenous
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<td>cytosolic PLA₂</td>
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<td>iPLA₂</td>
<td>Ca²⁺-independent phospholipase A₂</td>
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<td>Src homology 2 domain-containing transforming protein</td>
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<td>secreted protein acidic and rich in cysteine</td>
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<td>Src</td>
<td>sarcoma oncogene</td>
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<td>superficial zone protein</td>
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# WEIGHTS AND MEASURES

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CHAPTER 1 - INTRODUCTION

1.1 Articular Cartilage Morphology

Articular cartilage is an important contributor to synovial joint function. Joint contact areas must be large enough to transmit applied loads at pressures that materials of the joint can tolerate. Articular cartilage covers the ends of the diarthroidal joint bones and aids in spreading the load across the joint. It performs two main functions; first, to absorb stress by deforming under mechanical load and, second, to provide a smooth, load-bearing surface to permit low friction movement of the joint.

The composition of cartilage varies slightly from species to species, individual to individual and from joint to joint but, in general, it comprises the same three components: cells, matrix water and a macromolecular matrix network. Essentially, only one cell type is present, namely chondrocytes which are found embedded in a matrix of collagen and proteoglycan, making up under 10% of the tissue volume. Chondrocytes are responsible for organising the collagen, proteoglycans and non-collagenous proteins into a unique highly ordered structure, cartilage. Normal cartilage tissue possesses no blood supply to act as a source of nutrition, nor does it possess any nerves thus making it difficult for any damage to be perceived.

1.1.1 Chondrocytes

Articular cartilage contains a single sparse population of highly specialised cells known as chondrocytes. These cells comprise less than 10% of the tissue volume (Vanwanseele et al., 2002). Chondrocytes are completely surrounded by extracellular matrix (ECM) and are unable to contact one another via cell-cell interactions (Buckwalter and Hunziker, 1999). Chondrocytes from different cartilage zones differ in size, shape and metabolic activity but all share a complex relationship with the surrounding ECM. Each contains organelles necessary for matrix synthesis.

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(endoplasmic reticulum and the Golgi complex) as well as intracytoplasmic filaments, lipid, glycogen and secretory vesicles and some have microvilli extending into the matrix.

Articular cartilage chondrocytes differ in activity and function during skeletal development and growth and after maturity. Whilst growing, chondrocytes produce new tissue to expand and remodel the articular surface but, once mature, they do not significantly change the volume of the tissue but, instead, replace degraded matrix macromolecules, which may play a role in remodelling the articular surface (Buckwalter, 1995).

1.1.2 Articular Cartilage Zones

The structure of articular cartilage changes with depth from the joint surface. There are four distinct zones or layers referred to as the superficial or tangential zone, the middle or transitional zone, the radial or deep zone and the region of calcified cartilage (Figure 1.1). Although it is clear that these regions are distinct, exact boundaries cannot be defined. The matrices within the zones vary with respect to water, proteoglycan and collagen concentration and in aggregate size. The chondrocytes also differ in shape, size and orientation, as well as numerical density and metabolic activity (Buckwalter and Hunziker, 1999).

1.1.2.1 The Superficial Zone

The superficial zone is the thinnest of the four zones, comprising of two regions; an acellular layer and a deeper cellular layer, lying parallel to the articular surface. The former consists of a thin layer of amorphous material overlying a deeper sheet of fine fibrils with little polysaccharide. The acellular region, also known as the lamina splendens (Jurvelin et al., 1996) overlies a deeper sheet of fine fibrils containing low concentrations of polysaccharide (Buckwalter et al., 1988). The lower, cellular layer comprises cells that are flattened and ellipsoid in shape surrounded by a collagenous matrix. The cell’s major axes are orientated so
Figure 1.1 Articular cartilage zonal organisation. A) A cross section through full depth articular cartilage (www.dahweb. engr.ucdavis.edu/dahweb/126site/tidemark.htm). B) A pictorial representation of the zones present in articular cartilage (www.bartleby.com/107/68.html).
that they are parallel with the cartilage surface. The chondrocytes synthesise a matrix that has a high concentration of collagen but low concentrations of proteoglycan. Fibronectin and water concentrations are also highest in this zone (Buckwalter and Hunziker, 1999).

Normal structure and properties of the superficial zone are of great importance for the weight-bearing function of cartilage, since its morphology influences the wear and lubrication mechanisms operating between the joint surfaces, where its structure and composition effectively control both the compressive and tensile properties of the tissue as a whole (Jurvelin et al., 1996). Any damage to the superficial zone will compromise the mechanical properties of the tissue and may, therefore, initiate catabolic events leading to further degeneration and cartilage breakdown (e.g. in osteoarthritic joints).

Superficial zone protein (SZP) also known as PRG4 (proteoglycan 4) or lubricin (Jay et al., 2001) is a 345kDa, multi-functional domain proteoglycan, homologous to the precursor protein of a megakaryocyte-stimulating factor (MSF) (Merberg et al., 1993). SZP was originally isolated and purified by Schumacher et al., (1994) from culture media of superficial slices of bovine articular cartilage. The N-terminal domain of SZP has potential growth-promoting, cytoprotective and matrix-binding properties. SZP also has lubricating and cyto-protective properties provided by its mucin-like mid-domains and possible matrix binding and aggregating properties resident within the C-terminal domain (Flannery et al., 1999a).

SZP is specifically synthesised and secreted by articular cartilage superficial zone chondrocytes and does not appear to be present in either the transitional or deep zones. Chondrocytes exposed to cytokines or growth factors exhibit differential expression of SZP and it is possible that an alternatively spliced form is present in human osteoarthritic cartilage (Flannery et al., 1999a). Compressed tendon tissue has been reported to contain elevated levels of cartilage-like proteoglycans compared to tensional tendon and the tissue phenotype changes and becomes more articular cartilage-like with age (Perez-Castro and Vogel, 1999; Vogel and Heinegard, 1999).
1985). SZP has since been found exclusively, on the surface of compressed regions of tendon and increases with age (Rees et al., 2002), in keeping with the phenotypic changes.

1.1.2.2 The Transitional Zone

This area is many times thicker than the superficial zone, making up approximately 40-60% of the tissue content. As the name suggests the morphology and matrix composition of this region is intermediate between the superficial zone and the radial zone; the cells here are spheroidal in shape, have a higher concentration of synthetic organelles and synthesise a matrix with larger diameter collagen fibrils, a higher concentration of proteoglycans but lower water and collagen concentrations (Buckwalter and Hunziker, 1999).

1.1.2.3 The Radial or Deep Zone

In this region, spheroidal chondrocytes are aligned into columns perpendicular to the articular surface. Collagen fibres emerge from the underlying calcified region where they anchor themselves (Vanwanseele et al., 2002). This region has the largest diameter collagen fibrils, the highest concentration of proteoglycans and the lowest water concentration compared to the other zones (Buckwalter and Hunziker, 1999).

1.1.2.4 The Zone of Calcified Cartilage

This is a transition zone between the articular cartilage and the underlying subchondral bone. Here the cells have a much smaller volume and only contain very small amounts of ER and Golgi complexes. The chondrocytes are often completely surrounded by calcified cartilage and, hence, have very low metabolic activity.
1.2 Extracellular Matrix (ECM)

The extracellular matrix (ECM) can be thought of as an isotropic network of tangled collagen fibrils of indefinite length, trapping and restraining an unstructured interfibrillar matrix of highly hydrated proteoglycans. Within the ECM, collagen fibrils act as tension elements, while the swelling pressure of the interfibrillar matrix represents the compression element (Ottani et al., 2001). The ECM is arranged into compartments around the cells: pericellular (closest to the cell), territorial (extending around individual or groups of chondrocytes) and the interterritorial matrix (furthest away). Chondrocytes are responsible for producing the matrix. As well as collagens and proteoglycans, there is also a minor population of non-collagenous matrix components present within the ECM.

1.3 Glycosaminoglycans

Glycosaminoglycans (GAGs) are a specific class of biological molecule found in connective tissues of all organisms, covalently linked to proteoglycans; the major macromolecule of the ECM. Proteoglycans are very diverse molecules and, as well as their major role as structural components, they are also able to regulate many cellular events and physiological processes including cell proliferation, differentiation, cell-cell and cell-matrix interactions. This potential is thought to be largely due to the diversity of their GAG side chains e.g. GAG type, size composition, as well as substitution and protein structural domain arrangements.

GAGs are linear polysaccharides made up of repeating disaccharide units. Each unit consists of one hexosamine (D-galactosamine or D-glucosamine), which can be either N-acetylated or N-sulphated, and one uronic acid (D-glucuronic acid, GlcA, or L-iduronic acid, IdoA) or a neutral hexose (D-galactose, Gal) (Vynios et al., 2002). (See Figure 1.2 for an illustration of GAG structure).
Figure 1.2 The structures of the major cartilage glycosaminoglycans (adapted from Roughley and Lee, 1994). Chondroitin sulphate also has a non-sulphated form (C-0-S). GalNAc, N-acetyl galactosamine; IdoA, Iduronic acid; GlcA, glucuronic acid; GlcNAc, N-acetyl glucosamine; GlcNSO₃, glucosamine-N-sulphate; Gal, galactosamine; SO₄, sulphate group; CS, chondroitin sulphate; DS, dermatan sulphate; KS, keratan sulphate; HA, hyaluronic acid; HS, heparan sulphate.
The presence of sulphate and carboxylate groups on GAG chains gives these molecules a very high negative charge, a characteristic that confers a very high affinity to water.

GAGs may be implicated in disease for a variety of reasons i.e. i) a structural fault of the GAG chain may be responsible for a disease (e.g. as is the case with keratan sulphate damage in corneal macular dystrophy), ii) a defect may be present in a matrix protein associated with a GAG chain, or iii) it is possible there could be a deficiency in the biosynthetic or catabolic steps of a GAG molecule e.g. many types of cancer where GAG biosynthesis is impaired (Vynios et al., 2002).

At present, at least six GAGs have been identified; chondroitin sulphate (CS), dermatan sulphate (DS), keratan sulphate (KS), heparan sulphate (HS), heparin (all of which are sulphated to varying degrees) and hyaluronic acid (HA), the only known non-sulphated GAG. With the exception of heparin, all are present in articular cartilage (Roughley and Lee, 1994).

1.3.1 Chondroitin Sulphate and Dermatan Sulphate

Chondroitin sulphate (CS) is a linear polysaccharide composed of repeating disaccharide units of glucuronic acid and N-acetyl galactosamine, with sulphate groups present at either position 4 or 6 of the hexosamine (Roughley and Lee, 1994).

Dermatan sulphate (DS) differs due to the presence of iduronic residues rather than glucuronic residues. Iduronic acid is formed from glucuronic acid by the action of an epimerase (Malmström and Aberg, 1982) and hence DS can be considered as a modified form of CS. Sulphation of DS may also occur at the 2-position of iduronate residues.

1.3.2 Keratan Sulphate

Suzuki first identified keratan sulphate in 1939 in corneal extracts (Suzuki, 1939) and by the 1950s Karl Meyer had characterised this material as a linear polymer of lactosamine, 3Galβ1-
4GlcNAcβ1, sulphated at the C6 of both hexose moieties (Meyer et al., 1953). It is the only GAG not to possess an uronic acid residue. Instead, it is composed of repeating disaccharide units of galactosamine and N-acetyl glucosamine (for a review see Funderburgh, 2000). Keratan sulphate is found on proteoglycans within the ECM of articular cartilage.

### 1.3.3 Heparin and Heparan Sulphate

Heparin and heparan sulphate (HS) have distinctly different repeating disaccharides compared to CS and DS i.e. glucuronic acid and N-acetyl glucosamine. Some of the glucosamine residues may undergo a process of deacetylation and subsequent N-sulphation. The glucuronic residues are susceptible to epimerisation to iduronic acid (Roughley and Lee, 1994). Heparin is more negatively charged than HS, and also has the highest charge density of any known biological macromolecule, whilst HS is generally less sulphated and with a lower IdoA content (Vynios et al., 2002).

### 1.3.4 Hyaluronic Acid

Hyaluronic acid (HA) or hyaluronan as it is also known, is the only, known non-sulphated GAG i.e. none of its hydroxyl groups are esterified with a sulphate group. HA is a linear, and unbranched polymer, composed of a simple repeating disaccharide, N-acetyl glucosamine and glucuronic acid (Kakehi et al., 2003). Unlike other GAGs whose synthesis takes place in the Golgi, HA is synthesised at the plasma membrane of the cell. Once outside of the cell, HA can form complexes with hyaluronan-binding proteins such as aggrecan or versican. HA is widely distributed in the intercellular matrix of mammalian connective tissues and is, by far, the largest GAG with more than 2500 disaccharide units.
1.4 Proteoglycans of Articular Cartilage

Proteoglycans are ubiquitous cell-surface and secreted macromolecules that contain a core protein to which one or more highly anionic GAG chains are covalently linked (Hardingham and Fosang, 1992). Proteoglycans belong to a versatile family whose potential functions proceed from either the GAG chains they carry or from the specific regions of their protein cores. Proteoglycans contribute to maintaining an essential microenvironment for cell adhesion, migration and proliferation (Jollès, 1994).

Hyaline cartilage contains several well-characterised proteoglycans, several of which are discussed below. The largest in size and abundance is aggrecan, a proteoglycan that possesses over 100 CS and 50 KS side chains. The others are much smaller molecules and are able to interact with the collagens present within the ECM. Versican, decorin, biglycan, fibromodulin and perlecan are all present in articular cartilage. Type IX collagen can also be considered as a proteoglycan as its α2(IX) chain may bear a GAG chain (this will be discussed later, see section 1.5.3) (Roughley and Lee, 1994).

1.4.1 Aggregating Proteoglycans

‘Aggregating’ relates to the ability of these proteoglycans to interact non-covalently with hyaluronic acid to form large proteoglycan aggregates. Aggrecan is probably the best characterised member of this class due to its high abundance in cartilage, its ability to enable cartilage to reversibly resist compression and, when modified, it can cause many cartilage disorders. This family of aggregating proteoglycans also includes versican, neurocan and brevican, although the latter two are not known to be expressed in cartilage and thus will not be discussed further. Each member of the family has an amino-terminal globular domain that interacts with HA and a carboxy-terminal globular domain with lectin-like homology. Due to

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this, the aggregating proteoglycans are also known as the ‘hyalectins’ or ‘lecticans’ (Iozzo and Murdoch, 1996; Roughley, 2001).

1.4.1.1 Aggrecan

Aggrecan is a multidomain proteoglycan with several well-characterised functional regions. It is the major structural macromolecule in the cartilage matrix and is responsible for tissue resilience and elasticity. Human aggrecan is the product of a single gene residing on chromosome 15 (Iozzo et al., 1992). The gene consists of at least 15 exons and covers 50kb of genomic DNA (Doege et al., 1990), of which the exons encode about 9kb. The primary sequence of aggrecan has been determined in many species and all show common features.

The average molecular weight of aggrecan is 3MDa with 90% of its mass contributed to by approximately 100 CS and 50 KS chains, as well as, 8-10 shorter N- and O-linked oligosaccharides covalently linked to a core protein of 0.25 MDa (Vertel, 1995).

The core protein is made up of a series of disulphide-bonded domains that fold into two separate globular regions, G1 and G2, at the amino-terminus and a single globular region, G3, at the carboxy-terminus (Roughley and Lee, 1994). The G1 globular region has the ability to interact with HA and the link protein in the ECM. This interaction is very stable and, in effect, immobilises the aggrecan molecule. There are 3 disulphide-bonded loops present within the G1 domain, A, B and B'. Loop A shares homology with the immunoglobulin super family whereas loops B and B' share homology with each other and are known as proteoglycan tandem repeats (PTRs).

The G2 domain, separated from G1 by a linear interglobular domain (IGD) has two PTRs but exhibits no HA binding properties. The function of this region is still unknown. There is a large GAG substituted region composed of KS and CS domains extending between G2 and G3 at the carboxy-terminal of the core protein. This region is highly variable between species.

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Figure 1.3 Aggrecan structure. A) A single bovine nasal aggrecan molecule seen under high resolution using atomic force microscopy (Ng et al., 2003) B) Interactions between the G1 domain of aggrecan, link protein and hyaluronan. Also depicted are GAG and oligosaccharide-substitution sites and globular domains G2 and G3 (Vertel, 1995). C) Schematic showing the domain structure and proteolytic cleavage sites within the aggrecan molecule (Caterson et al., 2000).
Characteristic features are the presence of Ser-Gly recognition sequences for CS attachment and the presence or absence of Glu-Pro-rich hexapeptide repeats for KS substitution. The KS domain is not always present (e.g. in rat and mouse aggrecan) although some KS substitution can also be found within the IGD. However, CS domains are present and are divided into the CSI and CSII domains due to differences in the organisation of the sequence repeats.

The final globular domain, G3 is totally unrelated to G1 and G2 and exhibits sequence variation due to alternative splicing. It shows homology to other unrelated molecules such as an epidermal growth factor (EGF)-like domain, a lectin-like domain and a complement-regulatory protein (CRP)-like domain.

In the ECM of cartilage, aggrecan forms enormous link-protein-stabilised aggregates with HA that are restrained within a 3D-lattice work of type II collagen-containing fibrils associated with several minor collagens and glycoproteins (Vertel, 1995). The sulphated GAG chains, of aggrecan, represent a concentrated region of negative charges. This attracts positive counter ions that, in turn, attract water thus, maintaining a highly hydrated and resilient tissue.

1.4.1.2 Versican

Versican has been found to be present in cartilage, liver, intervertebral disc and the skin. It is a member of the large, aggregating proteoglycan family that share similarities of structure and also the ability to interact with hyaluronan. The human versican gene is found on chromosome 5 (Iozzo et al, 1992). The gene is composed of 15 exons encompassing over 90kb of genomic DNA (Naso et al., 1994).

Versican is, in fact, a splice variant of a much larger proteoglycan, PG-M. The core protein of PG-M is encoded by 15 exons; with a long central CS substituted region encoded by exons 7 and 8. These are susceptible to alternative splicing, with four variants known. Versican is the name given to the variant containing exon 8 only (Zimmerman et al., 1989).
Versican has a similar structure to aggrecan, with a core protein of 2389 amino acids. There are cysteine-rich regions present at both the amino- and carboxy-terminals, as well as a long central region where 15-17 negatively charged CS chains are substituted (Gutierrez et al., 1997). Versican, however, does not have an equivalent G2 globular domain. The amino-terminal G1 domain possesses hyaluronan binding capabilities whilst the carboxy-terminal region possesses EGF-like, CRP-like and lectin-like domains homologous to those of aggrecan (Sztrolovics et al., 2002).

1.4.1.3 Link Protein

This is not a proteoglycan as there are no GAGs attached to the core structure. However, it does possess one or two N-linked oligosaccharides near the amino-terminus. Thus it is a glycoprotein and is synthesised by the chondrocytes of the matrix. Link protein has a similar structure to the G1 domain of aggrecan. It possesses three disulphide-bonded loops, two of which maintain the ability to interact with hyaluronic acid and one molecule of aggrecan. This interaction results in the formation of large proteoglycan aggregates that give cartilage the ability to resist compression under load (Hardingham, 1979).

The link protein is encoded on human chromosome 5 (Osborne-Lawrence et al., 1990), by a gene with 5 exons that encodes a 339 amino acid protein (Roughley and Lee, 1994). Three isoforms of link protein can be isolated from human articular cartilage, termed LP1, LP2 and LP3 with molecular masses of 48, 44 and 41kD respectively (for a good overview see Hughes et al., 1992). Each of these forms is derived from the same protein core and the mass differences are attributed to their variations in N-linked oligosaccharide substitution (Baker and Caterson, 1979).
1.4.2 Non-aggregating Proteoglycans

'Non-aggregating' is the term given to proteoglycans which do not have the ability to directly interact with HA to form mass aggregates. Many non-aggregating proteoglycans are products of distinct genes and these include decorin, biglycan and fibromodulin. This family of molecules is characterised by its ability to interact with collagen (Roughley and Lee, 1994).

1.4.2.1 Decorin and Biglycan

Human cartilage has been shown to contain three dermatan sulphate proteoglycans – biglycan, decorin and epiphycan (not discussed) and in all cases the DS side chains are found in the amino-terminus of the core protein. Only decorin and biglycan are found in articular cartilage and are present throughout life, although biglycan shows evidence of age-related proteolytic processing (Roughley, 2001).

Decorin is the product of an 8-exon gene on chromosome 12 (Danielson et al., 1993), which encodes a protein of 329 amino acids. The protein has small disulphide-bonded domains near both its amino and carboxy termini with a long central leucine-rich region. Decorin possesses a single DS side chain (Roughley and Lee, 1994). The functional properties of decorin are related to its ability to interact with collagen fibrils, type I and type II. In vitro, decorin is able to influence both the rate of collagen fibrillogenesis and the fibril diameter (Vogel and Trotter, 1987).

In humans, biglycan is encoded by an 8-exon gene, found on the X chromosome (Fisher et al., 1991). The major difference from decorin is the presence of two GAG side chains, which may be either DS or CS. Biglycan, is not thought to interact with collagen fibrils or influence fibrillogenesis although its precise function is still unclear.
1.4.2.2. Fibromodulin

Fibromodulin was first isolated from bovine articular cartilage (Heinegard et al., 1986), and has been cloned and sequenced from bovine (Oldberg et al., 1989) and human (Antonsson et al., 1993) sources. Fibromodulin differs from decorin and biglycan in two major ways. Firstly it does not have a binding region for GAG at the amino-terminus but instead the core protein contains many sulphated tyrosine residues. Secondly, the molecule contains consensus sequences for four N-glycosylated keratan sulphates or oligosaccharides within a central leucine-rich region. It is thought that the role of fibromodulin is to limit the interaction between collagen fibrils, hence having an opposing role to decorin (Plaas et al., 1990).

1.5 Collagens of Articular Cartilage

To be characterised as a collagen, a molecule must contain a stretch of at least twenty or so amino acids of the sequence Gly-X-Y, where X and Y are often proline and hydroxyproline, respectively. This sequence is responsible for forming a characteristic triple-helix (Sandell et al., 1999). A triple-helical or 'collagenous' domain consists of three separate chains, termed α-chains, each of which contains the characteristic sequence of amino acids twisted in the form of a left-handed helix termed a polyproline type II helix (Ramachandran and Ramakrishnan, 1976). Three helical chains are then wrapped around each other in a higher-order rope-like fashion to produce the tight, triple-helical structure of the molecule (Traub and Piez, 1971). The molecule is further stabilised with the addition of interchain hydrogen bonds (Ramachandran, 1988).

Each collagen has been assigned a number (Roman numerals) generally reflecting the chronological order in which it was discovered (Bornstein and Sage, 1980). Different α-chains within the helix are designated α1, α2, or α3, although some collagens may be homotrimers of three of the same α-chains and others may be heterotrimers with three different chains. Furthermore, some molecules are made from two identical α-chains and one different. To
complete the nomenclature of a collagen molecule, the ratio in which the α-chains are present must also be included. For example collagen type II, is a homotrimer of three α1 chains and so is termed [α1(II)], and collagen type VI, a heterotrimer is termed α1(VI) α2(VI) α3(VI).

Collagens and large aggregating proteoglycans are the major components of the extracellular matrix of articular cartilage tissue. 50-60% of dry weight of articular cartilage is collagen and, in the adult, 90% of this is the ‘fibril-forming’ type II collagen (Kuettner, 1992). Altogether articular cartilage contains around ten distinct collagens; it has long been known that collagen types II, VI, IX, X, and XI are major components in cartilage (Thomas et al., 1994; Bruckner and van der Rest, 1994), although others, type I (Duance, 1983), type III (Wotton and Duance, 1994), type V (Eyre and Wu, 1987) and types XII and XIV (Watt et al., 1992), have also been reported to be present at low concentrations (Duance et al., 1999).

In articular cartilage, collagen types II, IX and XI come together to form heterotypic fibrils (Mendler et al., 1989). These are composed of the fibril-forming collagen type II, with type IX covalently bound to, and decorating the fibril surfaces, whilst type XI forms head-tail self cross-linked filaments that become integrated and cross-linked laterally onto or within the body of the collagen type II fibrils (Eyre, 2002) (Figure 1.4).

1.5.1 Collagen Type II

Collagens type II and XI are closely related in structure and belong to the sub-group of collagens termed fibril-forming collagens. Other members include types I, III and V. Each fibrillar collagen is secreted as a pro-collagen containing a large central triple-helical domain, linked to amino- and carboxy-terminal propeptides. These pro-collagens have higher molecular weights than the equivalent collagen molecules found within the ECM and, hence, cleavage and removal of the propeptides, by proteinases, must occur prior to fibril formation.

Molecules of the fibrillar collagens are found within striated fibrils. The most accepted model for
Figure 1.4 Diagram to show a heterotypic collagen fibril found in cartilage consisting of types II, IX and XI collagen (Duance et al., 1999). Abbreviations: NC, non-collagenous domain.

The packing of the molecules describes a 1/4 length staggered alignment of molecules with a short empty space between the amino-termini of one molecule and the carboxy-termini of the next (Gross, 1974) (see Figure 1.5).

Collagen type II is the most abundant collagen present in articular cartilage making up 90% of the total collagen present in adult tissue (Kuettner, 1992). Type II is a homotrimer composed of three identical α-chains, [α1(II)], (Miller, 1971) and is synthesised almost exclusively by chondrocytes. Two forms of collagen type II exist, IIA and IIB, generated by alternative splicing of exon 2 that encodes a cysteine-rich domain (Ryan and Sandell, 1990). Type IIA contains this domain but IIB does not. Type IIA and IIB can be expressed and translated but tissue distribution differs. Type IIB is restricted to cartilage, whilst IIA is found in less mature tissues e.g. embryonic spinal ganglion (Sandell et al., 1991).

Collagen type II is susceptible to cleavage by collagenase I (also known as matrix metalloproteinase 1, MMP-1), which cleaves at a single bond between residues Gly775 and Leu776.
of the triple helix leading to destabilisation of the helical conformation (Gadher et al., 1988). Other collagenases, MMP-8, MMP-13 and MMP-18 are also capable of cleaving collagen type II.

1.5.2. Collagen Type XI

Collagen type XI is another fibril-forming collagen that, together with types II and IX, is co-assembled into the heterotypic fibres characteristic of articular cartilage (Mendler et al., 1989). Collagen type XI contains a large amino-terminal noncollagenous domain in addition to a 300nm triple helix. It is a heterotrimer composed of three different types of α-chains,

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\( \alpha_1(\text{XI}) \alpha_2(\text{XI}) \alpha_3(\text{XI}) \) (Eyre and Wu, 1987). The first two chains are homologous to \( \alpha_1(\text{V}) \) and \( \alpha_2(\text{V}) \) respectively (Eyre and Wu, 1987) and, hence, the molecule is thought to be the structural analogue in cartilage to collagen type V. In addition, chain \( \alpha_3(\text{XI}) \) is identical to a highly glycosylated \( \alpha_1(\text{II}) \) chain and it is possible that both are products of the same gene (Furuto and Miller, 1983). Collagen type XI forms an interconnecting secondary filamentous network that could provide links between fibrils as well as running within the fibrils (Bruckner and van der Rest, 1994).

1.5.3 Collagen Type IX

Type IX is a member of the family of Fibril Associated Collagens with an Interrupted Triple helix (also known as the FACIT family). Collagen type IX is a heterotrimeric collagen, termed \( \alpha_1(\text{IX}) \alpha_2(\text{IX}) \alpha_3(\text{IX}) \). The molecule is only 190nm in length and comprises three triple-helical domains linked together by flexible regions and an amino-terminal globular domain. The molecule contains three collagenous domains (COL1-3) and four noncollagenous (NC1-4). These NC domains are susceptible to proteolysis to a higher degree than the helical domains. A major feature of this collagen is that it can also be described as a proteoglycan since the NC3 domain of the \( \alpha_2(\text{IX}) \) chain contains a single attachment site for a covalently bound CS chain (Huber et al., 1988; Vaughan et al., 1985).

1.5.4 Other Collagens

Type VI collagen is the main constituent of the beaded filaments found in many connective tissues. It has been found in cartilage localised to the pericellular environment of chondrocytes (Keene et al., 1988). Type VI is a heterotrimeric molecule made of three distinct chains, \( \alpha_1(\text{VI}) \alpha_2(\text{VI}) \alpha_3(\text{VI}) \). The major function of this collagen is to provide a protective fibrillar
meshwork around the chondrocytes. During osteoarthritis the levels of type VI are found to be much higher (Chang and Poole, 1996).

Type X collagen, a network forming collagen, is also found present in articular cartilage. It is mainly found in the region of calcified cartilage found in adult tissue, which separates the cartilage from the bone (Linsenmayer, 1991) and is only synthesised by hypertrophic chondrocytes. Type X is a homotrimer consisting of three identical \( \alpha_1(X) \) chains, each containing a triple-helical domain flanked by two non-collagenous regions. The central triple helical region of 460 amino acyl residues contains eight imperfections of the Gly-X-Y repetitive sequence. Two imperfections of the Gly-X-Y-X-Y type located near the extremities of the triple helix, result in two possible major sites of cleavage by matrix metalloproteinase-1 (MMP-1) (Welgus et al., 1990; and for review see Bruckner and van der Rest, 1994). Types I, III, V, XII and XIV have also been found at very low levels in articular cartilage. Type I collagen is found abundantly in skin, bone and tendon but is only a minor collagen present in articular cartilage, where it is located in the lamina splendens of the superficial cartilage zone. Type III collagen has been found in both normal and osteoarthritic human cartilage, also within the superficial zone and seems to exist in association with type I, co-existing within individual fibres. Type V collagen has a very high homology with type XI and, although the exact function is still unknown, it is thought to have a significant impact on the normal functioning of the joint.

Two fibril-associated collagens are also found present within articular cartilage, sharing structural and sequence homology to type IX, these are types XII and XIV. Both are homotrimeric molecules with interrupted triple-helical domains. In bovine cartilage, types XII and XIV can be found as chondroitin sulphate proteoglycans (Watt et al., 1992). The collagen family is fast growing and to date 27 members are known. See Table 1.1 for a summary of collagen types, structures and locations.
<table>
<thead>
<tr>
<th>FAMILY OF COLLAGENS</th>
<th>TYPE OF COLLAGEN</th>
<th>LOCALISATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibril-Forming</td>
<td>I</td>
<td>Skin, bone, tendon, IVD</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>Vitreous, cartilage, IVD</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>Skin, blood vessels, cartilage, IVD</td>
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<tr>
<td></td>
<td>V</td>
<td>As I and II</td>
</tr>
<tr>
<td></td>
<td>XI</td>
<td>As I and II</td>
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<td></td>
<td>XXVII</td>
<td>Stomach, lung, gonad, skin, tooth</td>
</tr>
<tr>
<td>Network-Forming</td>
<td>IV</td>
<td>Basement membrane, cornea</td>
</tr>
<tr>
<td></td>
<td>VIII</td>
<td>Descement’s membrane, endothelial cells</td>
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<tr>
<td></td>
<td>X</td>
<td>Calcifying cartilage</td>
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<tr>
<td>FACITs</td>
<td>IX</td>
<td>Vitreous, cartilage, IVD</td>
</tr>
<tr>
<td></td>
<td>XII</td>
<td>Skin, cartilage, IVD</td>
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<tr>
<td></td>
<td>XIV</td>
<td>As XII</td>
</tr>
<tr>
<td></td>
<td>XVI</td>
<td>Skin, lung, arterial smooth muscle cells</td>
</tr>
<tr>
<td></td>
<td>XIX</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>XX</td>
<td>Cornea, epithelium, skin, cartilage, tendon.</td>
</tr>
<tr>
<td></td>
<td>XXI</td>
<td>Heart, stomach, kidney, skeletal muscle</td>
</tr>
<tr>
<td>Beaded Filaments</td>
<td>VI</td>
<td>Most tissues</td>
</tr>
<tr>
<td>Anchoring Filaments for Basement Membranes</td>
<td>VII</td>
<td>Anchor stroma to basement membrane in skin, cornea</td>
</tr>
<tr>
<td>Transmembrane</td>
<td>XIII</td>
<td>Most tissues</td>
</tr>
<tr>
<td></td>
<td>XVII</td>
<td>Skin, muscles</td>
</tr>
<tr>
<td></td>
<td>XXIII</td>
<td>Prostate carcinoma cells</td>
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<td></td>
<td>XXV</td>
<td>Alzheimers diseased brain</td>
</tr>
<tr>
<td>New collagens</td>
<td>XV</td>
<td>Most basement membranes, placenta, muscle</td>
</tr>
<tr>
<td></td>
<td>XVIII</td>
<td>Most tissues esp. liver</td>
</tr>
<tr>
<td></td>
<td>XXII</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>XXIV</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>XXVI</td>
<td>Neonate testis and ovary</td>
</tr>
</tbody>
</table>

Table 1.1 The collagen family; classification and location. Abbreviations: FACIT, Fibril associated collagen with an interrupted helix; IVD, intervertebral disc.

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1.6 Other Extracellular Matrix Molecules

As well as proteoglycans and collagens, other molecules are also present within the extracellular matrix of cartilage. Firstly, elastin is present and found associated with collagen and proteoglycans. It is the protein responsible for the elastic properties of vertebrate organs and tissues. The molecule is very similar in structure to collagen. It has very high concentrations of glycine and proline although, unlike collagen, there is no hydroxyproline present. Mature elastin is an insoluble polymer constituted by several or more tropoelastin molecules covalently bound to each other by cross-links. In between the cross-linking domains, the hydrophobic segments exhibit high mobility and give the molecule its flexibility (Debelle and Tamburro, 1999).

Another molecule existing within the ECM is fibronectin. There are three forms of fibronectin; plasma fibronectin that is present in blood and bodily fluids as a soluble dimer, cell surface fibronectin which is found attached to the cell surface, and thirdly, matrix fibronectin which is composed of insoluble aggregates of fibronectin that are involved in cell attachment to substrates. Fibronectin is a di-sulphide-bonded dimer of 230-250kDa monomers that may become linked into high-order multimers in the insoluble extracellular matrix by interchain disulphide bonds. The fibronectin polypeptide chain is best described as a linear series of repeating modules with different functional properties (Armstrong and Armstrong, 2000).

Secreted Protein Acidic and Rich in Cysteine (SPARC) is an extracellular glycoprotein expressed by many different types of cells and is associated with development, remodelling, cell turnover and tissue repair (Yan and Sage, 1999). SPARC (also termed osteonectin) is a 34kDa glycoprotein belonging to a group of matrix-associated glycoproteins that mediate cell-matrix interactions but do not serve primarily structural roles (Brekken and Sage, 2000). It was first described as a major constituent of bovine and human bone and as a protein secreted by proliferating cells in vitro (Lane and Sage, 1994). It has since been shown to be a Ca\(^{2+}\) -binding glycoprotein expressed by many different cell types. It functions as a counter-adhesive protein, a

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modulator of growth factor activity and as a cell-cycle inhibitor. SPARC is known to be upregulated in tissues undergoing repair or remodelling due to injury or disease such as in arthritis and cancer.

A recently discovered protein present in the extracellular matrix is the cartilage intermediate layer protein (CILP), a 91.5kDa single chain protein whose expression is thought to be altered in osteoarthritis (Lorenzo et al., 1998). The protein has been found to be restricted in its tissue distribution to specific zones within cartilage (i.e. the intermediate or transitional zone). Chondrocytes in the middle layers of the tissue appear to produce the protein and deposit it in the interterritorial matrix whereas CILP has not been found in the superficial or deepest regions (Lorenzo et al., 1998). It's function is still unknown but it's specific localisation suggests possible roles in structural maintenance and in tissue repair.

Cartilage Oligomeric Protein (COMP) was first isolated and characterised from Swarm rat chondrosarcoma (Mörgelin et al., 1992) and subsequently from bovine and human articular cartilage (Di Cesare et al., 1994). COMP is an extracellular protein belonging to the thrombospondin family of proteins and is a 524kDa, pentameric, di-sulphide bonded, multidomain glycoprotein (Di Cesare et al., 2002). The importance of COMP has been demonstrated by the findings that mutations in COMP cause skeletal dysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (EDM1). PSACH and EDM1 are associated with severe disproportionate to mild short structure, joint deformities, early onset arthritis and hip replacement in early adulthood (McKeand et al., 1996) More than 50 mutations of COMP have been found in patients with these disorders (Deere et al., 2001).

Other important proteins found within the extracellular matrix are the matrilins. Four members of this family exist with matrilin-1 being the best known. It is known that matrilin-1 has the ability to bind with chondroitin sulphate side chains of aggrecan, and along with it's ability to
associate with collagen fibrils, this suggests a role in connecting various molecular aggregates in cartilage.

1.7 Osteoarthritis

In general, osteoarthritic (OA) diseases occur as a result of both mechanical and biological events that destabilise normal chondrocyte metabolism involving degradation and synthesis of articular cartilage extracellular matrix and also changes in subchondral bone metabolism. Although they may be initiated by multiple factors, including genetic, development, metabolic and traumatic, OA diseases involve all tissues of the diarthrodial joint. Ultimately OA diseases are manifested by morphologic, biochemical, molecular and biomechanical changes of both cells and matrix which leads to a softening, fibrillation, ulceration, loss of cartilage, sclerosis and eburnation of subchondral bone, osteophytes and subchondral cysts. When clinically evident, OA diseases are characterised by joint pain and tenderness, limitation of movement, crepitus, occasional effusion and variable degrees of inflammation without systemic effects (Kuettner and Goldberg, 1995).

Osteoarthritis is rapidly becoming one of the diseases to most frequently affect the elderly of the Western world and, with an increasingly aging-population, OA is becoming a huge problem. At present it is the most common joint disorder in the world and, in the United States, its prevalence is second only to ischaemic heart disease. OA is widely viewed as an age-related dynamic reaction pattern of a joint in response to insult or injury (Dieppe, 1998).

Most joints maintain their structural integrity throughout life, but arthritic disease can cause joint destruction and failure through loss of articular cartilage during OA or through invasion of both cartilage and bone as seen in rheumatoid arthritis (RA), which is discussed in section 1.8. In both cases, alteration and remodelling of all the joint surfaces occurs, especially at the interfaces between cartilage, calcified cartilage and subchondral bone (see Figure 1.6).
Figure 1.6 Schematic representation of the pathological changes associated with osteoarthritic joints after many years of degenerative change compared to normal synovial joints (Poole and Howell, 2001).
Cartilage matrix turnover is a process of synthesis and degradation that is balanced in the healthy individual. OA is thought to be caused when a shift of this equilibrium occurs, either due to a decrease in production of vital ECM components or an increase in catabolism. For example, matrix metalloproteinases and aggrecanases, the enzymes responsible for the degradation of both collagens and proteoglycans are found at increased levels in diseased cartilage (Creamer and Hochberg, 1997).

There are thought to be a number of stages throughout the osteoarthritic process. First, the cartilage-specific stage where there is an increase in matrix turnover and the chondrocytes divide. Cartilage degeneration is first seen at the articular surface in the form of fibrillation. Splits appear parallel to the surface, later penetrating the damaged cartilage and reaching the underlying subchondral bone (Poole and Howell, 2001). Second, a period of bone hypertrophy occurs, where osteophytes form at the bone edges. This is then followed by synovial membrane enlargement, leukocyte infiltration, inflammation, cytokine production and, finally, joint destruction (Feldmann and Brennan, 2000).

1.8 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects the peripheral synovial joints, leading to inflammation and swelling and, in turn, destruction of the underlying connective tissue. RA affects approximately 1% of the world’s population with half of this number experiencing the crippling form of the disease that results in cartilage and bone destruction (Billingham, 1999). The incidence of RA increases with age and is most common amongst those aged between 40-70 years (Lee and Weinblatt, 2001).

RA is thought to be influenced by genetic factors. Heritability studies have shown population predispositions to RA to be high (approximately 60%) and analysis has shown a higher disease
concordance among monozygotic twins (12-15%) compared with dizygotic twins (4%) suggesting the influence of genetic factors (MacGregor et al., 2000).

The predominant clinical features of RA are pain, stiffness and swelling of peripheral joints. An inflamed synovium is central to the pathophysiology of RA (Lee and Weinblatt, 2001). The synovium exhibits pronounced angiogenesis, cellular hyperplasia, an influx of inflammatory leucocytes, as well as changes in the expression of cell-surface adhesion molecules, proteinases, proteinase inhibitors and many cytokines (Feldmann and Brennan, 2000). Early in RA onset, tissue oedema and fibrin deposition are prominent and can manifest clinically as joint swelling and pain. Soon, the synovial lining becomes hyperplastic, commonly with a layer of 10 or more cells deep, consisting of macrophage-like and fibroblast-like synoviocytes. The sublining sees alterations in cell number and content also, with infiltration of mononuclear cells such as T cells, B cells, macrophages and plasma cells (Lee and Weinblatt, 2001) (Figure 1.7).

The cartilage and subchondral bone interface is the site of attack by an invasive pannus, a tissue formed from the synovium and periosteum interface. The pannus is said to represent the 'moving edge' of inflammation. An inflammatory or immune insult to the synovium is thought to stimulate proliferation, overgrowth and invasion of cartilage and bone. It is not entirely clear what initiates pannus formation but the initial inflammatory and immune reactivity within the synovial cavity would result in the generation of cell proliferative cytokines and growth factors. However, the continuing immunity and inflammation in RA is not required for the progressive erosion of cartilage and bone. It is still unknown why some patients have erosive RA and others do not exhibit this (Billingham, 1999).
Figure 1.7 Major anatomical features of an inflamed joint in rheumatoid arthritis (www.arc.org.uk/about_arth/booklets/6033/6033.htm).
1.9 Cartilage Proteoglycan Turnover

The loss of aggrecan from articular cartilage matrix is one of the earliest pathophysiological hallmarks of osteoarthritis and rheumatoid arthritis (Hughes et al., 1998). The resulting loss of sulphated glycosaminoglycan causes the functional and structural integrity of cartilage to be compromised, which leads ultimately to a tissue that is incapable of resisting the compressive loads applied to it during joint articulation (Catterson et al., 2000).

During the progression of degenerative joint disease there is a sustained loss of the cartilage proteoglycan, aggrecan, into the synovial fluid. Studies have revealed two major sites for aggrecan cleavage within the interglobular domain (IGD) of the protein core; at peptide bonds Asn$^{141}$ – Phe$^{142}$ (Flannery et al., 1992) and Glu$^{173}$ – Ala$^{174}$ (Sandy et al., 1992) (human sequence enumeration). It has been shown that cleavage of the Asn-Phe bond is attributed to the matrix metalloproteinases (MMPs), whilst Glu-Ala proteolysis is carried out by an activity termed 'aggrecanase'. Proteolysis within the IGD results in the separation of the GAG-bearing regions of aggrecan monomers from the amino-terminal hyaluronan-binding G1 domain and, hence, the loss of aggrecan from the cartilage matrix. Sites for aggrecanase activity have also been located within the carboxy-terminal region of the aggrecan molecule (see Figure 1.3C). Research has now led to the identification, purification and cloning of aggrecanase 1 and 2, two members of the A Disintegrin And Metalloproteinase domain with ThromboSpondin motifs (ADAMTS) family of proteins (ADAMTS 4 and 5, respectively) (Abbaszade et al., 1999, Tortorella et al., 1999).
1.10 Matrix Proteases

1.10.1 Matrix Metalloproteinases

The matrix metalloproteinases (MMPs) also known as matrixins, are a class of related, yet structurally distinct, zinc-dependent proteases. They were first discovered in connection to their ability to degrade extracellular matrix proteins such as collagen and elastin. Gross and Lapière (1962) first discovered the MMP activity in the tail of the metamorphosing tadpole. This enzyme is now known as Collagenase 1 or MMP 1. Since this discovery, a large family of up to 25 (numbered MMP-1 to MMP-29) closely-related MMPs has been characterised, of which 23 are found in humans (Somerville et al., 2003). The family can be subdivided into four main groups that differ in size and substrate specificity. These groups are known as stromelysins, collagenases, gelatinases and membrane-type metalloproteinases (Cawston, 1998). Collectively, MMPs are able to degrade all components of the extracellular matrix.

The MMP family shares a number of characteristics; all MMPs contain homologous sequences, they are all secreted as proenzymes that must be activated by proteolytic cleavage or treatment with mercurial compounds and they all contain zinc at their active site as well as a secondary zinc atom needed to maintain a stable conformation. As well as the previous factors, MMPs are inhibited by a class of natural inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs) which all act at neutral pH and require calcium for activity (Cawston, 1998). MMPs play a key role in the normal physiology of connective tissue during development, morphogenesis and wound healing, but their unregulated activity has been implicated in numerous disease processes including arthritis, tumour cell metastasis and atherosclerosis (Brew et al., 2000). Growth factors (such as epidermal growth factor and fibroblast growth factor), hormones and cytokines (such as IL-1 and TNF) will stimulate the synthesis and secretion of MMPs while agents such as IL-4, IL-13 and IL-10 are all able to lower expression levels. The
sites of enzyme activation are mainly the cell surface and the extracellular space and the main control factors are activation of the proenzyme or zymogen and inhibition by TIMPs.

The primary structures of MMPs comprise several domain motifs (Figure 1.8). At the amino-terminal end of the molecule there is a propeptide domain of approximately 80 amino acids with a conserved PRCG(V/N)PD amino acid sequence. The conserved cysteine (C) within this sequence ("cysteine-switch") is responsible for ligating the catalytic zinc to maintain the latency of pro-MMPs (Van Wart and Birkedal-Hansen, 1990). The next important domain is the catalytic domain (about 170 amino acids) that contains a zinc-binding motif HexxHxxGxxH and fragments but are specific to different collagens. MMP-13 has an affinity to collagen type II and is, therefore, important in articular cartilage degradation. Gelatinases are responsible for a conserved methionine, which forms a unique ‘met-turn’ structure (Bode et al., 1993). A linker peptide, rich in proline connects this domain with the next. This domain is a carboxy-terminal haemopexin-like domain of approximately 210 amino acids, that has an ellipsoidal disk shape with a four bladed β-propeller structure (4 antiparallel β-strands and an α-helix) (reviewed by Nagase and Woessner, 1999).

The stromelysins have a broad substrate specificity including proteoglycans, laminin and fibronectin. The three collagenases; interstitial collagenase (MMP-1), neutrophil collagenase (MMP-8) and collagenase-3 (MMP-13), differ in their substrate specificity. Each cleaves fibrillar collagens at a single site to produce specific quarter and three-quarter length collagen cleaving denatured collagens left behind by the action of the collagenases, as well as, types IV and V collagen and elastin.
Figure 1.8 Domain arrangements of vertebrate metalloproteinases (adapted from Nagase and Woessner, 1999; Alexander, 2002).

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1.10.2 ADAMS

ADAMs are a group of metalloproteinases so-called because they are proteins with a Disintegrin and Metalloproteinase domain. These proteins are multidomain, transmembrane proteins up to 750 amino acids in length. They are made up of a pro-domain and the metalloproteinase, disintegrin, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane and cytoplasmic tail domains (Primakoff and Myles, 2000) (Figure 1.9). There is evidence to suggest that each domain has a functional or structural role in at least one ADAM. The pro-domain is thought to play a role in blocking protease activity (Roghani et al., 1999; Loechel et al., 1999), the metalloproteinase domain controls protease activity (Roghani et al., 1999; Loechel et al., 1998), the disintegrin domain and the cysteine-rich domain both play roles in adhesion (Yuan et al., 1997; Iba et al., 1999) whilst the EGF-like domain is thought to stimulate membrane fusion, for example the EGF-like domains of fertilin α and β (ADAM 1 and 2) have a function in sperm-egg binding and fusion (Chen et al., 1998).

At present there are at least 34 known members of the ADAM family. 17 of these have a zinc-dependent metalloproteinase active site and the correct amino acid sequence to be a functional proteinase, HexGHxxGxxHD (where x represents any amino acid) (Moss et al., 2001) the rest are probably lacking in metalloproteinase activity. Some of these ADAMs proteins have been detected in a wide variety of tissues including cartilage, and expression has been shown to vary depending on cell environment and exposure to catabolic stimuli, which suggests a role in cartilage matrix metabolism (Flannery et al., 1999b). Others have been found exclusively or predominantly in the testis suggesting roles in spermatogenesis and fertilisation. An example of an ADAM family member is TNFα-converting enzyme (TACE or ADAM-17) that is involved in proteolytic release of the extracellular domains of cell surface transmembrane growth factor molecules such as TNFα (Moss et al., 2001).
Figure 1.9 The domain structure of an ADAM family member (adapted from Moss et al., 2001; Primakoff and Myles, 2000).
1.10.3 ADAMTS

'ADAMTS' describes a Disintegrin-like and Metalloprotease domain with Thrombospondin type 1 motifs, a novel family of proteases found in both mammals and invertebrates. Members are distinguished from ADAM family members by the multiple copies of thrombospondin type 1 (TSP1) motifs they carry. TSP1 motifs have the ability to interact with the ECM and can anchor themselves to glycosaminoglycans, possibly targeting the protein to discrete regions of the ECM and may modulate the activity of these proteinases by binding to selective sites on substrate molecules (Flannery et al., 1999b).

At present there are up to 20 known members of the ADAMTS family (Arner, 2002). They are extracellular matrix (ECM) proteins with a wide range of activities and functions distinct from members of the ADAM family. ADAMTS proteins are soluble ECM proteases whose known substrates are other ECM proteins. They are secreted proteins and consist of a pro-domain, a metalloprotease domain and a disintegrin domain. They lack the ADAM’s cysteine-rich, EGF-like, transmembrane and cytoplasmic tail domains but instead have thrombospondin type 1 (TSP-1) repeat motifs (Bornstein, 1992) present on the carboxy-terminal side of the disintegrin domain (Primakoff and Myles, 2000; Tang, 2001).

Aggrecan loss from articular cartilage by proteolytic cleavage is a major pathophysiological event in osteoarthritis and rheumatoid arthritis. Cleavage sites have been located within the interglobular domain of aggrecan and, have been shown to be cleaved by MMPs and a proteolytic activity termed 'aggrecanase'. Biochemical purification of two aggrecanases has shown that they are members of the ADAMTS family. Using neoepitope antibodies, aggrecanase activity has been followed and, hence, two aggrecanases have been purified (see Figure 1.10). Aggrecanase 1 (ADAMTS-4) and Aggrecanase 2 (ADAMTS-5 also known as ADAMTS-11) have been identified in IL-1 stimulated bovine nasal cartilage conditioned media (Tortorella et al., 1999, Abbaszade et al., 1999). ADAMTS-4 and ADAMTS-5 cleave aggrecan within the
Figure 1.10 Domain organisations of ADAMTS proteins, ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) (adapted from Cal et al., 2002)
IGD between residues Glu$^{373}$ and Ala$^{374}$ (Sandy et al., 1992) and not at the MMP-sensitive site between Asn$^{341}$ and Phe$^{342}$ (Flannery et al., 1992). Further work on ADAMTS-4 has revealed four additional sites for cleavage within the chondroitin-sulphate-rich region between G2 and G3 of the aggrecan core protein (Tortorella, 2000).

1.10.4 Tissue Inhibitors of Metalloproteinases

An important mechanism for the regulation of matrix metalloproteinase activity is via binding to a family of homologous proteins referred to as the tissue inhibitors of metalloproteinases (TIMPs). So far, four members of the TIMP family are known in humans (TIMPs 1-4). Mammalian TIMPs have a relative molecular weight of about 21kDa and are two-domain molecules. Each has an amino-terminal domain of approximately 125 amino acids and a smaller carboxy-terminal domain of about 65 residues (Brew et al., 2000). Each domain is stabilised by three disulphide bonds.

All connective tissues contain members of the TIMP family (Cawston, 1998) and expression is controlled during tissue remodelling and physiological conditions to maintain a balance in extracellular matrix metabolism. If there is a shift in equilibrium resulting in uncontrolled matrix turnover, diseases such as arthritis, cancer and cardiovascular conditions may arise.

Differences between the inhibitory properties of the four TIMPs have been reported. For example, TIMP-2 and TIMP-3, unlike TIMP-1, are effective inhibitors of the membrane-type MMPs. Another example is TIMP-2 that binds tightly to the zymogen form of MMP-2 forming a complex that is important in the cell-surface activation of pro-MMP-2 (Brew et al., 2000). In general, TIMPs bind tightly to all active MMPs with a 1:1 stoichiometry (Cawston, 1998).

Experiments have shown that TIMP-3 is also a potent inhibitor of both ADAMTS-4 and -5, although this is not true for TIMPs-1 and -2. This suggests that TIMP-3 may also act as a specific regulator of aggrecanase activity (Gendron et al., 2003; Kashiwagi et al., 2001).
Therefore the TIMPs play an important role in controlling connective tissue breakdown by blocking activated MMP action and preventing activation of the pro-enzymes.

1.11 Inflammatory Mediators

Inflammation is a highly complex process, a response to an insult or injury, and a series of interconnecting pathways and reactions. Mediators of inflammation can be divided into classes, of which lipid mediators, cytokines and nitric oxide will be discussed here. Many lipid mediators, which can influence inflammation, are formed from the free (non-esterified) fatty acids, which have, themselves, to be released from complex glycerolipids.

1.11.1 Phospholipase A₂

The phospholipase A₂ (PLA₂) super family consists of at least 19 enzymes (Kudo and Murakami, 2002) with the ability to catalyse the hydrolysis of the central (sn-2) ester bond of membrane glycerolipids (van Deenen and de Haas, 1963). The PLA₂ action is considered to be the rate-limiting step in the release of free fatty acids (e.g. arachidonic acid [AA]) and lysophospholipids and, hence, the subsequent formation of prostaglandins (PGs) and leukotrienes (LTs) as well as other bioactive lipids, via the cyclooxygenase (COX) and lipoxygenase (LOX) pathways.

Free (non-esterified) fatty acids are thought to be important as regulators of various cellular processes and are the precursors of eicosanoids (PGs and LTs). Lysophospholipids have roles in cell signalling, phospholipid remodelling and membrane perturbation (Six and Dennis, 2000).

PLA₂ enzymes are broadly categorised into ‘intracellular’ and ‘extracellular’ and three major groups exist; secretory PLA₂ (sPLA₂), cytosolic PLA₂ (cPLA₂) and Ca²⁺-independent PLA₂ (iPLA₂). cPLA₂ is a 85kDa, Ca²⁺-dependent enzyme with selectivity for AA at the sn-2 position (Yedgar et al., 2000). After stimulation, cPLA₂ translocates to the cell membrane whereupon it releases free fatty acids.

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PLA₂ enzymes are found to be present at high concentrations in joints with inflammatory disease such as arthritis. It has also been shown that PLA₂ induces inflammation when injected into healthy animals and that pro-inflammatory cytokines such as IL-1 and TNF can induce PLA₂ activity (Bomalski and Clark, 1993).

1.11.2 Cyclooxygenases

Cyclooxygenase (COX), also known as prostaglandin H synthase (PGHS), catalyses the first two steps in the formation of inflammatory prostaglandins (PGs) and other eicosanoids. Two isozymes are commonly known to exist, COX-1 and COX-2, although recently, Chandrasekharan et al., (2002) isolated and sequenced a third distinct isozyme, COX-3, as well as two smaller COX-1–derived proteins (partial COX-1 proteins). These will be discussed further in Section 1.11.2.1.

COXs are heme-containing proteins that exhibit both cyclooxygenase and peroxidase activities (Hla et al., 1999). The cyclooxygenase (bis-oxygenase) reaction occurs in a hydrophobic channel in the enzyme core and here arachidonic acid (AA) plus two molecules of oxygen are converted to prostaglandin G₂ (PGG₂) (see Figure 1.11). The peroxidase reaction is the reduction of PGG₂ to PGH₂ by the removal of two electrons and occurs independently or during cyclooxygenase catalysis at the heme-containing active site located near the protein surface (Smith et al., 2000; Gurr et al., 2002).

COX-1 and COX-2 are constitutive and inducible enzymes, respectively. COX-1 is found in most cells and tissues and is thought to play a role in maintaining and regulating normal physiological processes such as gastric secretion and kidney function. COX-2, however, is known to be induced by pro-inflammatory mediators such as growth factors, cytokines and tumour promoters and has been found at high levels in chronic inflammatory conditions, for example arthritis, peritonitis and colon cancer. COX-1 was initially purified in 1976 (Miyamoto...
Figure 1.11 The cyclooxygenase reaction in schematic form leading to the biosynthesis of cyclic endoperoxide, PGH₂ (taken from Gurr et al., 2002).
et al., 1976) and then cloned in 1988 (Merlie et al., 1988), whereas COX-2 was not discovered until much later (O’Banion et al., 1991). Both the COX proteins have a molecular weight of 71kDa and are approximately 60% identical. The COX-1 and -2 genes encode enzymes located in the luminal compartment of the endoplasmic reticulum and the nuclear envelope, respectively. The genes for both enzymes are highly regulated; COX-1 is generally induced by cell quiescence and differentiation whilst COX-2 is an immediate-early gene induced by a wide variety of stimuli, as previously mentioned. The COX-1 gene resembles a housekeeping gene, lacking a TATA box whilst the COX-2 gene is compact and contains a TATA box as well as several inducible enhancer elements or transcriptional regulatory sequences including C/EBP, AP-2, CRE and NFκB sites (Appleby et al., 1994 and for a review see Hla et al., 1999).

Although both enzymes utilise arachidonic acid (AA) as a substrate, COX-2 is able to utilise a much wider range of fatty acids also. The reason for this is due to a major difference in protein structure between the two isozymes. The catalytic site of COX-1 is much smaller than that of COX-2. Several amino acid substitutions including the replacement of an isoleucine in COX-1 with a valine in COX-2 leads to an increase in the relative volume of the active site consistent with the substrate promiscuity of this enzyme (Picot et al., 1994).

COX-1 and COX-2 are the targets for the non-steroidal anti-inflammatory drugs (NSAIDs), for example aspirin and ibuprofen. NSAIDs compete with AA for binding to the cyclooxygenase catalytic site and, hence, are able to reduce inflammation pain and fever. In fact, once bound, aspirin will acetylate an active-site serine. COX-1 is completely inhibited by aspirin acetylation whereas COX-2, with its larger active site, is still able to metabolise AA after aspirin treatment. An unpleasant gastro-intestinal toxicity side effect experienced by some patients has been attributed to the inhibition of COX-1 (Lipsky, 2001). This finding has led to the design of specific COX-2 inhibitors. Drugs such as Cerebrex and Valdecoxib (Chavez and DeKorte, 2003) are able to specifically inhibit COX-2 without affecting the normal housekeeping role of COX-1.

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COX-2 is constitutively expressed in the brain and kidney but, as yet, it is not known whether inhibition will lead to any long-term side effects on the normal functioning of these tissues (Bolten, 1998).

1.11.2.1 COX-3 and the Partial COX-1 Proteins

Acetaminophen is one of the world's most popular analgesic/antipyretic drugs. It is classified as an NSAID although it is known to have little anti-inflammatory action. It does, however, reduce pain and fever. The mechanism of action of acetaminophen is unclear but previous work by Flower and Vane (1972), showed that cyclooxygenase activity was inhibited at higher levels in the canine brain than in other tissues. This suggested that the drug might preferentially inhibit different isozymes of COX. Work using COX-1 and -2 has showed that neither were sensitive to acetaminophen, thus suggesting a possible third COX enzyme.

Dan Simmons' group (Chandrasekharan et al., 2002) studied canine cerebral cortex and have isolated and sequenced a third distinct COX isozyme, COX-3, as well as two smaller COX-1 derived proteins known as the partial COX-1 proteins (PCOX-1a and PCOX-1b). COX-3 and PCOX-1a are formed by intron retention; a poorly understood form of alternative splicing. Each is encoded by the COX-1 gene but retain intron 1. PCOX-1a is identical to COX-3 except for a 219aa in-frame deletion across exons 5-8 of the COX-1 mRNA. PCOX-1b is identical to PCOX-1a but lacks intron 1 (see Figure 1.12). Activity and inhibition studies have shown COX-3 to be present at high concentrations in the cerebral cortex of dog as well as in human brain and aorta, and it has approximately 20% of the activity of COX-1. PCOX-1a, however, lacks the majority of its cyclooxygenase and peroxidase active sites and has no known COX activity (Chandrasekharan et al., 2002). It has also been shown that COX-3 is preferentially inhibited by analgesic/antipyretic drugs, including acetaminophen, dipyrone, diclofenac and phenacetin over either COX-1 or COX-2 (see also Warner and Mitchell, 2002).

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**Figure 1.12** A schematic representation of the domains of COX-3 and PCOX-1a in comparison to COX-1. COX-2 has a similar domain structure to COX-1 and PCOX-1b is identical to PCOX-1a except that it lacks intron 1 (adapted from Chandrasekharan *et al.*, 2002). Abbreviations: aa, amino acids; bp, base pair; EGF-like, epidermal growth factor-like.
1.11.3 Prostaglandins

Eicosanoids comprise a diverse group of oxygenated metabolites of polyunsaturated fatty acids and include prostanoids (the various prostaglandins, prostacyclin and thromboxane), the leukotrienes, and various epoxy, hydroperoxy, and hydroxy fatty acids (Gurr et al., 2002). Biosynthesis of each eicosanoid begins with oxygenation of the fatty acid precursor. For the prostaglandins (PGs), thromboxane and prostacyclin this step is catalysed by COX activity that converts AA to PGG₂ (discussed in section 1.11.2). The peroxidase activity of COX then reduces PGG₂ to PGH₂ (Kulmacz, 1998).

Prostaglandins are derivatives of fatty acids, all have a five-membered cyclopentane ring with two aliphatic side chains attached. Prostaglandins are able to bind to cell-surface receptors and transduce signals via G-proteins. These small lipid molecules play a large role in inflammation but are also known to regulate numerous other processes in the body, including kidney function, platelet aggregation and neurotransmitter release (Harris et al., 2002).

The numbering system for PGs includes a number to indicate the amount of double bonds within its structure. Since ring closure involves the participation of two of the fatty acid precursor's double bonds, a PG such as PGE₂ will have originated from a tetraenoic PUFA (e.g. AA) whilst PGE₁ will have been formed from a pentaenoic precursor.

1.11.3.1 Prostaglandin E₂ (PGE₂)

PGH₂ is produced by both COX-1 and COX-2 isoforms and is the common substrate for a series of specific synthases that produce PGD₂, PGE₂, PGF₂α, PGI₂ and TxA₂ (Tilley et al., 2001) (see Figure 1.13). Probably, the most well known and the best studied of these is PGE₂. PGE₂ is produced by many cells of the body including fibroblasts, chondrocytes, macrophages and some malignant cells and exerts its actions by binding to one (or more) of four subtypes of receptor (named EP₁, EP₂, EP₃, and EP₄) (Harris et al., 2002). The receptors are rhodopsin-type receptors.
Figure 1.13 Conversion of PGH₂ into various eicosanoids (taken from Gurr et al., 2002).
containing seven hydrophobic transmembrane domains, coupled though their intracellular sequences to specific G-proteins with different second messenger signalling pathways (Breyer et al., 2001). Various inflammatory agents (or PGE₂ itself) are able to regulate the expression of EP receptor subtype on different cells enabling PGE₂ to have very specific effects on various tissues. PGE₂ has both pro- and anti-inflammatory effects as well as immunosuppressive roles. Pro-inflammatory roles include induction of fever, increasing vasodilation and enhancing pain and edema (swelling) that can be caused by other agents such as histamine. PGE₂ is also able to suppress the production of TNFα and interleukin-1, suppress lymphocyte production and natural killer cell activity and inhibit the production of interleukin-2, hence having anti-inflammatory and immunosuppressive functions. PGE₂ also promotes IgE production, a mediator of allergic inflammation, by B-lymphocytes (reviewed by Calder, 2003b). Studies have shown that PGE₂ has the ability to inhibit 5-lipoxygenase activity and, hence, the generation of leukotrienes of the 4-series e.g. LTB₄ and induce 15-lipoxygenase activity and production of lipoxin A₄, a known inflammation “stop signal” (Levy et al., 2001). So although PGE₂ possesses distinct pro-inflammatory actions it appears to also be able to mediate the resolution of inflammation by effecting eicosanoid production. Other roles associated with PGE₂ include modulation of sleep/wake cycles, facilitation of human immunodefeciency virus (HIV) replication, elevation of cAMP levels, stimulation of bone resorption and effects on thermoregulation (www.RnDSystems.com).

1.11.4 Lipoxigenases
Lipoxigenases (LOXs) are a class of non-heme iron dioxygenases that catalyse the hydroperoxidation of polyunsaturated fatty acids (PUFAs) in plants, animals and microorganisms. In additional to common natural substrates, such as linoleic acid and arachidonic acid, a wide variety of other PUFAs can act as substrates for these enzymes.
Mammalian LOXs catalyse the hydroperoxidation of arachidonic acid, initiating the synthesis of two families of potent physiological effectors, leukotrienes (LTs) and lipoxins (Prigge et al., 1996). LOXs are composed of a single polypeptide chain approximately 75-80kDa in animals. The proteins have an amino-terminal β-barrel domain as well as a larger catalytic domain containing a single atom of non-heme iron. The iron is liganded to conserved histidines and to the carboxyl group of a conserved isoleucine at the carboxy terminal of the protein. LOX enzymes are usually in the ferrous (inactive) form when isolated and oxidation to the active ferric enzyme is required for catalysis (Brash, 1999).

The nomenclature of mammalian LOXs is based on positional specificity and includes the carbon atom to which the dioxygen binds in products of AA, for example 12-LOX oxygenates AA at carbon-12. In mammalian cells there are three major types of LOX; 5-LOX, 12-LOX and 15-LOX (see Figure 1.14).

1.11.4.1 5-LOX

5-lipoxygenase (5-LOX) was originally found in rabbit leukocytes in connection with the discovery of leukotrienes (Samuelsson et al., 1987) but is generally found in neutrophils and is known to catalyse the oxidation of carbon-5 of AA. 5-LOX knockout mice have been shown to have normal physiological function but are inept in manifesting acute inflammatory responses (Chen et al., 1994).

5-LOX and the integral membrane protein, 5-lipoxygenase activating protein (FLAP) are required for the cellular synthesis of leukotrienes in intact cells. 5-LOX translocates to the nuclear envelope in response to a variety of stimuli and catalyses the oxygenation of AA to 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and its subsequent dehydration to leukotriene A₄ (LTA₄). This can be metabolised to LTB₄ by LTA₄ hydrolase, or to
Figure 1.14 Flow diagram showing the formation of inflammatory mediators from fatty acid via phospholipase A₂, cyclooxygenase and lipoxygenase action and their products (adapted from Harris et al., 2002). Abbreviations: HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; NSAIDs, non-steroidal anti-inflammatory drugs.
LTC₄, LTD₄, and LTE₄ by a pathway initiated by glutathione S-transferase (Reddy et al., 2003).

FLAP is an 18kDa member of a protein superfamily designated as the membrane-associated proteins in eicosanoid and glutathione metabolism, or MAPEG (Peters-Golden and Brock, 2003). The FLAP molecule contains three potential transmembrane domains and a hydrophobic carboxy-terminal tail. It is known that leukotriene synthesis only occurs when both 5-LOX and FLAP are co-expressed (Vickers, 1995). It appears that FLAP acts as a fatty acid transfer protein that 'presents' the substrate to 5-LOX on the nuclear membrane (Mancini et al., 1993). So far, it seems that FLAP is unique to 5-LOX and no equivalent proteins have been identified for the other lipoxygenases.

1.11.4.2 12-LOX

12-LOX was the first member of the lipoxygenase family characterised and was discovered in human and bovine platelets. It has two major tissue locations and, hence, has two classifications; 'platelet-type' 12-LOX and 'leukocyte-type' 12-LOX. The former is only active with free AA to convert it to 12-HPETE (12S-hydroperoxy-5,8,10,14-eicosatetraenoic acid), whereas the latter, leukocyte-type has a broader substrate range and acts on both free and esterified fatty acids to form both 12- and 15-HPETE (15S-hydroperoxy-5,8,10,14-eicosatetraenoic acid). 12-HPETE can later be reduced to 12-HETE (12S-hydroxy-5,8,10,14-eicosatetraenoic acid) (reviewed by Yoshimoto and Yamamoto, 1995).

1.11.4.3 15-LOX

15-LOX introduces molecular oxygen at carbon atom 15 of arachidonic acid to form 15-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid (see Kühn and Thiele, 1995 for review). 15-LOX was first detected in rabbit reticulocytes but has since been detected in a wide variety of

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mammalian cells and tissues such as reticulocytes, eosinophils, airway epithelial cells and monocytes. The arachidonic acid metabolites formed in this pathway antagonise inflammatory responses and counteract the proinflammatory effects of the 5-LOX pathway (Conrad, 1999).

1.11.5 Leukotrienes

Leukotrienes, products of the 5-LOX pathway, are potent biologically-active molecules. Those derived from AA have been best studied and are implicated as mediators of hypersensitivity reactions and inflammation. These inflammatory mediators are involved in the pathology of many diseases such as asthma, allergic rhinitis, rheumatoid arthritis and inflammatory bowel disease (Reddy et al., 2003). The name ‘leukotriene’ is used for two reasons. First, they were initially found in leukocytes and second, because they contain a conjugated triene chromophore (Kühn and Borngräber, 1999). Leukotrienes are abbreviated to LT followed by a capital letter (A, B, C, D, or E), indicating the chemical structure of the substituents, and a subscript that indicates the number of double bonds (the same as the fatty acid used for its synthesis) e.g. LTB₄.

1.11.5.1 Leukotriene B₄ (LTB₄)

LTB₄ is a potent lipid pro-inflammatory mediator, synthesised when 5-LOX enzymatically transforms arachidonic acid into the epoxide intermediate, LTA₄ that is then hydrated to yield LTB₄. LTB₄ binds to membrane receptors and causes leukocyte adhesion and chemotaxis. It is also known to induce aggregation, granule enzyme release and superoxide anion generation by neutrophils. There is an overproduction of LTB₄ in many inflammatory disorders (Henderson, 1994). LTB₄ generation provides amplification of neutrophil-mediated inflammation. LTB₄ can also bind to the peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor that acts as a transcription factor to induce the expression of enzymes involved in fatty acid oxidation.

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(Lam and Austen, 2000). Abnormal production has been seen in cases of inflammatory bowel disease, psoriasis, rheumatoid arthritis and gout.

1.11.6 Interleukin-1

There are two species of interleukin-1 (IL-1), which are both products of separate genes and exhibit a limited structural homology. The two isoforms, IL-1α and IL-1β are acidic and neutral, respectively. Each is synthesised as a 31kDa pro-IL-1 molecule, lacking signal peptides and are then cleaved to a mature 17kDa molecule. Pro-IL-1α, IL-1α and IL-1β are biologically active but pro-IL-1β is not. The IL-1 family consists of the two agonists, IL-1α and IL-1β, two receptors, the biologically active IL-1RI and the inert IL-1RII, as well as an antagonist IL-1Ra. The IL-1 signalling mechanisms are described in section 1.15.3.

There is considerable evidence to show that IL-1 is an important mediator of connective tissue degradation in arthritic conditions (Oyajobi and Russell, 1992, Dinarello, 1996). IL-1 is produced by various cell types and induces inflammation by activating synovial cells, endothelial cells, lymphocytes and macrophages to produce various chemokines, cytokines and inflammatory mediators such as IL-1, IL-6, TNFα, IL-8 and COX-2. It can cause infiltration of inflammatory cells into sites of inflammation, increase blood vessel wall permeability and induce fever. IL-1 also promotes synovial growth and activates synovial cells and osteoclasts to produce metalloproteinases and collagenases that cause erosion of the bone and cartilage of joints (Iwakura, 2002).

1.11.6.1 IL-1α

IL-1α is distinct from the other member of the IL-1 family, IL-1β. IL-1α triggers the same IL-1 receptor and many of its biological effects are similar to IL-1β but, in humans, IL-1α is mainly intracellular. The precursor is as biologically active as the mature cleaved form. Calpain, a
calcium-activated cysteine protease is responsible for precursor cleavage. The biological activities of IL-1α are characterised as proinflammatory (Dinarello, 2000a).

1.11.6.2 IL-1β

IL-1β is converted from its immature precursor to its mature active form, intracellularly, by the cysteine protease, IL-1β-converting enzyme (ICE), also known as caspase-1 (Dinarello, 1998). It is thought that other proteases e.g. cathepsin G and proteinase 3, can convert the precursor to its active form (Adkison et al., 2002). IL-1β is a secreted molecule (via ICE), whereas IL-1α is primarily a cell-associated molecule. There are no biological activities carried out by IL-1β that are not carried out by IL-1α, and vice versa. Again, the activities are characterised as pro-inflammatory (Dinarello, 2000b).

1.11.7 Interleukin-6 and Interleukin-8

IL-6 is a multifunctional cytokine produced by a variety of different cell types of lymphoid and non-lymphoid origin. TNF-α is a strong inducer of IL-6 gene expression and production by several cell types. Rheumatoid synovial cells produce IL-6 spontaneously and IL-1 and TNF-α can enhance this. This molecule plays a role in a wide range of responses, acute-phase reactions and haematopoiesis. IL-6 is a 186 amino acid protein, glycosylated at positions 73 and 172 (Hirano et al., 1986). IL-8 is a pro-inflammatory cytokine produced by various types of cells upon stimulation with inflammatory stimuli and exerts a variety of functions on leucocytes. Inhibition of IL-8 by specific antibodies has shown a reduction in neutrophil infiltration into acute inflamed tissues (Iizasa and Matsushima, 2000).
1.11.8 Tumour Necrosis Factor

Tumour necrosis factor (TNF) exists in two forms, TNF-α and TNF-β, with a high degree of sequence homology between the two (Oyajobi and Russell, 1992). Both forms have a similar activity to IL-1 although they are not structurally related and they bind to different cell-surface receptors. Some of the functions shared with IL-1 are the ability to inhibit collagen synthesis and the induction of prostaglandin and metalloproteinase synthesis. Although TNFα is required for protection against bacterial infection, it is also involved in cell growth modulation, response to viral infection, immune system regulation, septic shock, autoimmune diseases, rheumatoid arthritis, inflammation and diabetes (Aggarwal et al., 2000). TNF-α or cachectin as it was previously known, is a 17kDa peptide produced mainly by the activated macrophages or monocytes. It is produced spontaneously in synovial tissue cells in arthritic conditions. The presence of IL-1 and TNF-α in synovial fluid or inflammatory joint diseases are major factors in the destruction of articular cartilage and subchondral bone. TNF-α is a potent stimulator of angiogenesis, which may be involved in the vascularization of the invading pannus seen in rheumatoid arthritis (Oyajobi and Russell, 1992).

1.11.9 Nitric Oxide

Nitric oxide (NO) is a diatomic free radical with a high affinity for heme iron, sulphhydryl or thiol groups, superoxide anion and molecular oxygen (Jang and Murrell, 1998). Due to the rapid reactivity between NO and these groups, the former has a very short half-life (a matter of seconds). NO is small in size, has a neutral charge and, hence, is able to pass through membranes very easily, making it an excellent candidate to act as a signalling and effector molecule. Chondrocytes are the major source of NO in diarthroidal joints and are also responsible for synthesising inducible nitric oxide synthase (iNOS) (see section 1.11.10). NO can play roles in many cellular events of inflammatory response such as vascular flow, leukocyte migration and

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can activate cytotoxic effects on resident cells resulting in tissue injury to the host. NO has been shown to suppress proteoglycan synthesis (Taskiran et al., 1994), increase metalloproteinase activity (Murrell et al., 1995), prevent collagen chain cross-linking and, hence, reduce tensile strength (Cao et al., 1997) and increase COX-2 and PGE\(_2\) production (Manfield et al., 1996).

1.11.10 Inducible Nitric Oxide Synthase

NO is synthesised by a family of nitric oxide synthases that are encoded by three distinct NO genes; endothelial (ecNOS) and neuronal (ncNOS) are both named after the cells they were discovered in, both are constitutive enzymes and both require calcium influxes for instant activity. In contrast the third synthase, the inducible nitric oxide synthase (iNOS) is Ca\(^{2+}\) independent. All three isoforms utilise the amino acid L-arginine as their substrate (Jang and Murrell, 1998). iNOS can be induced by inflammatory cytokines such as IL-1\(\beta\) and TNF\(\alpha\). Macrophages, neutrophils, hepatocytes, chondrocytes and smooth muscle cells all have the ability to express iNOS.

1.12 Fatty Acids

Dietary fat is an essential macronutrient in the diet of all animals. It provides not only a source of energy, but also, hydrophobic components for the synthesis of complex lipids (Jump et al., 1996). Fatty acids are also used in the synthesis of signalling molecules such as prostanoids or in protein modification. A major function of polyunsaturated fatty acids is as precursors for the synthesis of eicosanoids such as prostaglandins, a class of compounds that exert hormone-like effects in many physiological processes. Other studies have led to the discovery that fatty acids can affect gene expression leading to altered metabolism, cell growth and differentiation.

A fatty acid is composed of a long hydrocarbon chain (‘tail’) and a terminal carboxy group (or ‘head’). The carboxy group is normally ionised (i.e. COO-) under physiological conditions. Most

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fatty acids found in nature are straight chain and have an even number of carbon atoms (14-24) and are either saturated (all carbon-carbon bonds are single) or unsaturated (with one or more double carbon-carbon bonds). Unsaturated fatty acids can have a single double bond (monounsaturated) or many double bonds (polyunsaturated). Unsaturated fats are more abundant in nature than saturated fatty acids.

Some fatty acids are not synthesised by mammals but are necessary for normal growth and life, these are termed the ‘essential fatty acids’. These include linoleic and α-linolenic acids that are obtained from plant constituents of the diet. Arachidonic acid is not normally found in higher plants but can be synthesised by mammals from linoleic acid via γ-linolenic and dihomo-γ-linolenic acids. Another fatty acid, α-linolenic acid can be converted via destauuration and elongation to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in animals (Calder et al., 2003b). EPA and DHA are also present in high concentrations in oily fish and fish oils and, hence, can be ingested directly from these sources.

1.13 *n*-3 and *n*-6 Polyunsaturated Fatty Acids

Humans can synthesise most lipids necessary for good health. Exceptions are the fatty acids belonging to the *n*-3 and *n*-6 families of polyunsaturated fatty acids (PUFA). Studies over the last two decades have shown that the amount and type of these fats consumed in the diet can profoundly influence biological responses (Alexander, 1998). There are three families of unsaturated fatty acids that influence immune and other cellular responses. These are named with respect to the location of the first double bond. For example, *n*-3 fatty acids (also known as omega-3) have their first double bond between the 3rd and 4th carbons (numbering from the methyl terminus), *n*-6 (omega-6) have the first double bond between carbons 6 and 7, and *n*-9 (omega-9) have theirs between carbons 9 and 10 (see Figure 1.15). The important dietary *n*-3 and *n*-6 fatty acids are polyunsaturated whereas oleate (a monounsaturated fatty acid) is,
Figure 1.15 A summary of fatty acid structure showing the positions of the key carbon-carbon double bonds e.g. n-3 fatty acids are so called as their first carbon-carbon double bond from the methyl end of the chain is found between carbons 3 and 4.

quantitatively, the only important n-9 fatty acid (Table 1.2).

Members of the n-6 family of PUFAs are usually more prevalent in dietary foods than the n-3s, although clinical deficiencies of n-6 are more common. α-Linolenic acid (n-3) is found in vegetable oils such as soybean oil and canola oil and can be converted by animals into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) via desaturation and elongation reactions and, in the case of DHA, a retroconversion step.

Oily fish e.g. mackerel, herring, and extractable fish oils (e.g. cod liver oil) all have very high concentrations of n-3 PUFAs, mostly present in the form of EPA and DHA. n-6 polyunsaturated fatty acids are present at high concentrations in plant oils such as sunflower oil. Linoleic (n-6)

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<table>
<thead>
<tr>
<th>COMMON NAME</th>
<th>NUMERICAL NAME</th>
<th>FATTY ACID SUBCLASS</th>
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<tr>
<td>Palmitic acid</td>
<td>16:0</td>
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<tr>
<td>α-Linolenic acid</td>
<td>α-18:3</td>
<td>n-3 polyunsaturated</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
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<tr>
<td>(EPA)</td>
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<tr>
<td>Docosahexaenoic acid</td>
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<tr>
<td>(DHA)</td>
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<tr>
<td>Linoleic acid</td>
<td>18:2</td>
<td>n-6 polyunsaturated</td>
</tr>
<tr>
<td>γ-Linolenic acid</td>
<td>γ-18:3</td>
<td>n-6 polyunsaturated</td>
</tr>
<tr>
<td>Dihomo γ-linolenic acid</td>
<td>20:3</td>
<td>n-6 polyunsaturated</td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td>20:4</td>
<td>n-6 polyunsaturated</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1</td>
<td>n-9 monounsaturated</td>
</tr>
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</table>

Table 1.2 Some important fatty acids. The table shows common names, numerical names and the subclass of fatty acid to which each belongs.

can be converted to arachidonic acid via the same mechanisms of desaturation and elongation.

The Western diet has an imbalance between the two groups of PUFAs with up to twenty times more n-6 PUFA, and this is thought to be responsible for the production of much higher levels of inflammatory leukotrienes and prostaglandins than if n-3 PUFAs were acting as the major substrates.

Linoleic acid is converted to arachidonic acid (AA), which is the precursor to the 2 series of prostaglandins (PGs) e.g. PGE₂ and the 4 series of leukotrienes (LTs) e.g. LTB₄. EPA is the precursor to the 3 series of PGs and 5 series of LTs, all of which have considerably lower inflammatory activity than AA metabolites (see Figure 1.16). Moreover, a diet rich in n-3 PUFAs, will result in a decrease in inflammatory mediator production due to competition for the enzymes involved in the metabolism of both n-3 and n-6 PUFAs. The consequences of this explain observations previously made during studies of Eskimos diets and health (Kromann and

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Figure 1.16 Synthesis of eicosanoids from different precursor fatty acids. Arachidonic acid is the precursor to the 2-series of prostaglandins (PGs) and the 4-series of leukotrienes (LTs) while eicosapentaenoic acid is the precursor to the 3-series of PGs and the 5-series of LTs (adapted from Calder and Zurier, 2001)
Green, 1980). Their diet is enriched with high amounts of oily fish and they exhibit a much lower incidence of inflammatory joint diseases. Although n-3 PUFAs are important in the diet, they cannot substitute for n-6 PUFAs. Both are essential, but there must be a correct balance between the two. Present suggestions are for a ratio of approximately 4:1 (n-6:n-3) in the diet but this depends on physiological requirements as well as the exact nature of the PUFAs in the diet (Cunnane, 2003).

1.14 Previous n-3 PUFA and Disease Research

A vast array of clinical and scientific research is focused on finding alternative therapies for reducing the symptoms and progression of inflammatory diseases. Many pharmacological treatments are anti-symptomatic but costly and ineffective at treating the underlying disease (for a recent review see Curtis et al., 2004). For example in the UK, in the year 2000, £341 million was spent on prescribing drugs for arthritis and £405 million spent on hip and knee replacements (www.arc.org.uk). n-3 PUFAs such as eicosapentaenoic acid are found at high concentrations in oily fish and fish oils such as cod liver oil. The beneficial effects of cod liver oil on patients suffering from sore and inflamed joints has been recorded as far back as 1783. A paper published in the London Medical Journal (Percival, 1783) describes the effects of ‘Oleum Jecoris Aselli’, or cod liver oil, in chronic rheumatism. Research has also focused on epidemiological studies of the Inuit (Eskimos). A low incidence of arthritis and musculoskeletal diseases have been noted among these populations and has been attributed to a fishy diet rich in n-3 PUFA (Kromann and Green, 1980). The beneficial effects of n-3 PUFAs have also been highlighted in many other diseases such as atherosclerosis, inflammatory bowel diseases (i.e. Crohn’s disease), rheumatoid arthritis, psoriasis and many others (see Table 1.3).

Many studies have been carried out into the effects that n-3 PUFAs are having on inflammation and the immune response. n-3 PUFAs are known to have beneficial effects on inflammatory
<table>
<thead>
<tr>
<th>DISEASE</th>
<th>LITERATURE CITED</th>
<th>Clinical Trial or Research</th>
<th>Review Article</th>
</tr>
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<td>Inflammatory Bowel Diseases</td>
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**Table 1.3** A summary of evidence for the beneficial effects of n-3 polyunsaturated fatty acids on pathology and symptomology of a variety of inflammatory diseases (gathered using Gil, 2002; Volker and Garg, 1996; Heller et al., 2003).
cytokine production, adhesion molecule expression and inflammatory gene expression in disease conditions (for a comprehensive review Calder, 2003a). Studies using animal models have shown that n-3 PUFAs (namely eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) can inhibit the production of IL-1β and TNFα by cultured monocytes (Calder, 1997) and the production of IL-6 and IL-8 by cultured venous endothelial cells (de Caterina et al., 1994). Cell culture studies have also shown that fish oils supplementation decreased the production of IL-1β, TNFα and IL-6 in rodent macrophages (Yaqoob and Calder, 1995). n-3 PUFAs and dietary fish oils also effect the ability of murine macrophages and human venous endothelial cells to bind to various surfaces by inhibiting the production of E-selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (de Caterina et al., 1994). Recent work indicates that n-3 PUFAs can also influence transcription factor expression through direct actions on intracellular signalling pathways for example the NFκB transcription factor and the peroxisome proliferator-activated receptors (PPARs). The NFκB pathway is known to induce the expression of many pro-inflammatory genes, such as COX-2, iNOS, ICAM-1, VCAM-1, E-selectin, TNFα, IL-1β, IL-6 and MMPs (see Calder, 2003a). n-3 PUFAs have also been shown to inhibit the 5-lipoxygenase (5-LOX) pathway and, hence, the metabolism of arachidonic acid into leukotrienes in both monocytes and neutrophils (Lee et al., 1985). Recently, work has shown that these same fatty acids can inhibit the mRNA expression of degradative enzymes (ADAMTS-4, ADAMTS-5, MMP-3 and MMP-13) and inflammatory factors (COX-2, IL-1, TNFα, 5-LOX and 5-LOX-activating protein [FLAP]) in bovine and human OA chondrocytes (Curtis et al., 2000; 2002).

Many clinical trials using n-3 PUFAs in the treatment of arthritis have also been carried out. The first placebo controlled human study used capsules containing high concentrations of EPA and DHA. The patients recorded beneficial improvements in physical factors such as tender joints and morning stiffness (Kremer et al., 1985). Many trials have since followed, with varying
results, which can be attributed to many factors such as \(n-3\) or \(n-6\) content in the normal diet, PUFA type, dose and mixture within the capsules given to the patients or the level of arthritis onset. Few trials have investigated the underlying molecular mechanisms affected by \(n-3\) PUFA supplementation.

Thus, \(n-3\) PUFAs are potentially anti-inflammatory agents and, hence, maybe of great importance in the therapeutic treatment of inflammatory diseases such as osteoarthritis, rheumatoid arthritis, asthma as well as various cancers, among others.

1.15 Signalling Pathways

In addition to its role as an energy source and its effects on membrane lipid composition, dietary fat may have profound effects on gene expression leading to changes in metabolism, growth and cell differentiation (Jump et al., 1996). In mammals, several transcription factors (for example \(NF\kappa B\), peroxisome proliferator activated receptors [PPARs], sterol regulatory element binding proteins [SREBP], as well as others) have been identified as either direct or indirect targets of PUFAs (Jump and Clark, 1999).

In mammals, the expression of many genes has been shown to be modulated by PUFAs in both positive and negative manners. Theoretical mechanisms have been put forward for PUFA regulation of gene transcription. PUFAs or their metabolites can:

- induce a cascade of events leading to a covalent modification of a transcription factor e.g. phosphorylation, redox state or proteolytic modification that leads to an altered transactivation capacity,
- directly bind to and activate transcription factors,
- modify mRNA stability,
- influence the transcription rate of a transcription factor, hence changing its \textit{de novo} synthesis (Duplus et al., 2000).
1.15.1 NFκB Pathway

NFκB has a role in the pathogenesis of diseases characterised by increases in the host immune and inflammatory response e.g. asthma, rheumatoid arthritis (RA), inflammatory bowel disease, atherosclerosis, Alzheimer’s disease, as well as a variety of cancers (Yamamoto and Gaynor, 2001). In RA, for example, NFκB is over-expressed in the inflamed synovium where its activity may enhance recruitment of inflammatory cells and production of pro-inflammatory mediators like IL-1, IL-6, IL-8 and TNF-α (Tak and Firestein, 2001). NFκB comprises a family of inducible transcription factors that serve as important regulators of host immune and inflammatory response. Each member of the family contains a conserved N-terminal region called the Rel-homology domain (RHD) within which lies the DNA-binding and dimerisation domains and the nuclear localisation signal (NLS) (May and Ghosh, 1998). At least five members of this family exist; p65, c-Rel, RelB, p50/p105 and p52/p100. The first three are produced as transcriptionally active proteins, whilst the others are produced as non-active precursors of 105 and 100kDa products, which require cleavage to smaller, active proteins.

NFκB is able to increase the expression of specific cellular genes. These include genes encoding at least 27 different cytokines and chemokines, receptors involved in immune recognition e.g. MHC, antigen presenting proteins and receptors required for neutrophil adhesion and migration (Pahl, 1999). The cytokines produced are themselves able to activate the NFκB pathway enabling amplification of the inflammatory response.

NFκB can also stimulate the expression of enzymes involved in inflammation, such as the inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2), both of which have products (nitric oxide and prostaglandins respectively) that contribute to the pathogenesis of inflammation (Pahl, 1999). In the resting cell, NFκB proteins are localised in the cytoplasm and are associated with a family of inhibitor proteins known as IκB. Seven IκB proteins are known; IκBα, IκBβ, IκBγ, IκBe, Bcl-3, p100 and p105, all of which comprise several distinct
domains, including ankyrin repeats that are needed for IκB interactions with NFκB and an amino-terminal domain that is a target for inducible phosphorylation and ubiquitination of IκB and a carboxy terminal domain important in regulating IκB turnover. The IκB proteins bind to NFκB and, hence, block the nuclear localisation signal. Upon stimulation by a variety of factors such as cytokines (e.g. IL-1 or TNF), LPS, UV light, or viral infection, the IκB is degraded and NFκB can translocate to the nucleus (see Figure 1.17).

Phosphorylation of IκB proteins is mediated by IκB kinases (IKKs). IKK exists as a complex of at least two kinase subunits; IKKα and IKKβ. It has been shown that IKKβ is the crucial kinase involved in activating the NFκB pathway while IKKα plays an accessory role (Zandi and Karin, 1999). IKK phosphorylates the IκB proteins on two serine residues of the amino-terminus. This then leads to the ubiquitination of two amino-terminal lysine residues and so targets IκB for degradation by the 26S proteosome. Once NFκB is free of its IκB subunits it is able to translocate to the nucleus where it is able to bind to specific elements in the promoter regions of target genes to activate specific gene expression (Yamamoto and Gaynor, 2001).

1.15.2 Mitogen Activated Protein Kinase (MAPK) Pathways

The mitogen activated protein kinases (MAPKs) are a family of well-conserved serine/threonine kinases with a molecular weight between 38-55kDa. Members of this family have a central role in a variety of protein kinase cascades. MAPK signalling pathways play an important role in signal transduction in eukaryotic cells where they modulate many cellular events including mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement and apoptosis as well as cell and neuronal differentiation (Murray, 1998). These evolutionary conserved pathways are organised into 3-kinase modules consisting of a MAPK, an activator of MAPK (MAPK kinase, MAPKK or MEK), and a MAPK kinase kinase (MAPKKK or MEKK) (see Figure 1.18). At least three distinct MAPK signal transduction
**Figure 1.17** Schematic showing simplified NFκB signalling pathway. An extracellular stimuli leads to the activation of NFκB that, in turn, translocates to the nucleus where upon the transcription factor is able to activate specific gene expression (section 1.15.1). Abbreviations: IkBα, inhibitor of NFκB; IKK, IκB Kinase.
Figure 1.18 A schematic showing the three major mitogen activated protein kinase (MAPK) cascades; ERKs, JNKs, and p38 leading to the activation of various transcription factors and gene transcription. Abbreviations: P, phosphate group; TF, transcription factor; IL-1, interleukin-1; TAK-1, TGF-β activated kinase.

pathways exist in mammals, each named after the MAPK associated with it: i) the extracellular signal-regulated kinases (ERK 1 and 2), ii) the c-Jun N-terminal kinases also known as the stress-activated protein kinases (JNKs or SAPKs) and iii) the p38 kinases. It is also likely that many more pathways exist, yet to be discovered.

MAPKs are proline-directed serine/threonine kinases that are activated by dual-phosphorylation in response to diverse extracellular stimuli. The dual phosphorylation occurs in activationdomain of MAPKs on the threonine (T) and tyrosine (Y) residues in the sequence pTXpY, where X is glutamic acid in ERKs, proline in JNKs and glycine in p38 and p represents a phosphate group.

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The dual phosphorylation is facilitated by dual-specificity MAPKKs, which in turn are activated by serine/threonine phosphorylation by MAPKKKs. This kind of three-tier regulatory cascade not only conveys information to target effectors, but also coordinates incoming information from parallel signalling pathways, allows for signal amplification, generates a threshold and a sigmoid activation profile, is subject to many inactivation mechanisms and accommodates a variety of mechanisms for control of subcellular localisation (Cobb, 1999).

1.15.2.1 The Extracellular Signal-Regulated Kinases

By far the best studied of all MAPKs are the extracellular signal-regulated kinases (ERKs). Mammalian ERK1 and ERK2 and their upstream kinases, MEK1 and MEK2, are stimulated by a variety of growth and differentiating factors (e.g. epidermal growth factor [EGF], platelet derived growth factor [PDGF]) through receptor tyrosine kinases (RTKs), heterotrimeric G-protein-coupled receptors or cytokine receptors (Hunter, 1995).

Ligands stimulate receptor tyrosine kinase activity, leading to enhanced tyrosine kinase autophosphorylation. Adaptor molecules containing SH2 or other phosphotyrosine binding domains (e.g. Grb2, Shc) bind to the receptors and are linked, via the adaptor’s SH3 domains, to a proline-rich region of the guanine nucleotide exchange protein, son of sevenless (Sos) (reviewed by Schlessinger, 1994; Cobb, 1999). Sos is responsible for enhancing GDP release and GTP binding to the Ras oncogene (a membrane anchored GTPase). The GTP-Ras complex then binds to the MAPKKK (in this case, Raf) bringing it to the plasma membrane where protein kinase activity is increased and the cascade activated. Upon activation, Raf phosphorylates the dual specificity MAPKKs (MEK1 and MEK2) (Hunter, 1995). These then activate the MAPKs, ERK1 and ERK2 by dually phosphorylating a threonine and a tyrosine residue, Thr\textsuperscript{201}-Glu-Tyr\textsuperscript{205} for ERK1 and Thr\textsuperscript{185}-Glu-Tyr\textsuperscript{197} for ERK2 (Kyriakis and Avruch, 2001). Once activated, ERKs
can translocate to the nucleus, where they phosphorylate target transcription factors, thereby regulating their activity (Peyssonnaux and Eychene, 2001).

1.14.2.2 The c-Jun N-Terminal Kinases

UV radiation, protein synthesis inhibitors and cytokines are among the activators of the JNK cascade (Han et al., 1999). The c-Jun N-terminal kinases (JNKs), also known as the stress-activated protein kinases (SAPKs), are activated by phosphorylation of their threonine and tyrosine residues at positions 183 and 185 respectively. This reaction requires the preceding activation of upstream MAPKKs (MKK4 and MKK7). MKK4 is known to activate both JNKs and p38 kinases whilst MKK7 is a specific activator of JNK only (Foltz et al., 1998). Once activated, JNKs can phosphorylate a number of cytoplasmic and nuclear proteins. Of particular interest are the transcription factors such as c-Jun, ATF-2 and Elk-1. It is thought that JNKs are the only kinases with the ability to phosphorylate the serines 63 and 73 of the amino-terminal of c-Jun, hence their classification (Mielke and Herdegen, 2000; Paul et al., 1997). As well as nuclear substrates, JNKs can phosphorylate cytoskeletal proteins, p53, Bcl-2 and the glucocorticoid receptor, all of which are located in the cytoplasm.

1.15.2.3 The p38 Kinases

p38 kinases are widely expressed in many tissues and are activated by dual phosphorylation on threonine 180 and tyrosine 182 within the pTGYsite (see section 1.15.2). The phosphorylation reaction is mediated by the MAPKKs; M KK3 and M KK6. It is unclear whether the MKK4 activation of both JNKs and p38 has a relevant function under physiological conditions or just in vitro (Mielke and Herdegen, 2000). Stimulants of this cascade include lipopolysaccharide, proinflammatory cytokines and cellular stresses such as osmotic shock (Han et al., 1999). It is thought that p38 kinases and JNKs work synergistically. p38 may contribute to AP-1 activity.
through induction of c-Jun and c-Fos via phosphorylation of ATF-2 and Elk-1, as in the case of JNKs. It is also known that p38 can phosphorylate CHOP, a member of the C/EBP family of transcription factors that mediates the effect of cellular stress on growth and differentiation (Wang and Ron, 1996).

1.15.3 The IL-1 Pathway

Interleukin-1 (IL-1) is a multifunctional pro-inflammatory cytokine (Auron, 1998), two isotypes of which exist. IL-1α and IL-1β are both synthesised as precursors with leading sequences that are cleaved by specific cellular proteases to form active 17kDa molecules (Dinarello, 1997). Two primary IL-1 cell surface binding proteins exist; IL-1 receptor I (IL-1RI) and IL-1 receptor II (IL-1RII). IL-1RI transduces a signal whereas IL-1RII binds IL-1 but does not transduce a signal. It seems that IL-1RII acts as a sink for IL-1 and has been termed a ‘decoy’ receptor. After binding with IL-1, IL-1RI forms a complex with the IL-1 receptor accessory protein (IL-1RacP). This then leads to the recruitment of the IL-1 receptor associated kinase (IRAK) to the complex via the adaptor protein MyD88. Phosphorylated IRAK then interacts with a member of the TRAF family, TRAF-6 (TNF-receptor-associated factor-6), which then initiates signalling cascades (such as the NFκB or MAPK pathways) involved in inducing the expression of many genes (Thomas et al., 2002) (see Figure 1.19).

Once bound to cells, IL-1 is capable of inducing several biochemical events. In general, multiple protein phosphorylation and activation of phosphatases can be observed after just five minutes. Phosphorylation of PLA₂ activating protein also occurs, leading to a rapid release of arachidonic acid (Dinarello, 1997).
Figure 1.19 The IL-1 signalling system: the molecules and signal transduction (adapted from Iwakura, 2002). Abbreviations: IL-1RacP, IL-1 receptor accessory protein; IL-1RI, IL-1 receptor I; IL-1RII, IL-1 receptor II; IRAK, IL-1 receptor associated kinase; MyD88, an adaptor protein; TRAF, TNF receptor associated factor.
1.15.4 Peroxisome Proliferator Activated Receptors

Isseman and Green first identified peroxisome proliferator activated receptors (PPARs) in 1990. PPARs are single polypeptide nuclear receptors that have a structure similar to other members of the steroid-thyroid supergene family of nuclear receptors (Jump and Clark, 1999). Three isoforms exist, PPAR-α, -β and -γ, and are encoded for by three separate genes (Duplus et al., 2000). PUFAs are a class of compounds known to activate PPARs. Studies have shown that the eicosanoids are also PPAR activators, e.g. prostaglandin J2 (PGJ2) binds to PPARγ (Jump and Clark, 1999) and leukotriene B4 (LTB4) binds to PPARα, causing their activation (Duplus et al., 2000). PPARs have emerged as important factors in the regulation of gene transcription by PUFAs and, thus, have diverse effects that lead to changes in metabolism and cell differentiation. Unsaturated n-3 and n-6 fatty acids that have undergone significant desaturation and elongation, along with other peroxisome proliferators, induce peroxisomal β-oxidation and many of the mRNAs encoding peroxisomal enzymes (Jump and Clark, 1999).

1.15.5 Nuclear Factor of Activated T-cells

The nuclear factor of activated T-cells (NFATs) family of mammalian transcription factors is regulated by calcium and known to play a key role in activating gene expression in T-lymphocytes during antigenic stimulation as well as playing roles in the function and development of neuronal, cardiac and other cells (Cyert, 2001). NFAT transcription factor activation is controlled primarily through its localisation. The proteins are cytosolic in resting cells but upon activation, calcium levels rise and calcineurin (Ca2+/calmodulin-activated phosphatase) dephosphorylates NFAT and causes its translocation to the nucleus. Here, NFATs work with AP-1 transcription factors to promote gene expression (Cyert, 2001).
1.16 Aims of the Project

Previous studies (Curtis et al., 2000; 2002) have shown that the expression and activity of degradative enzymes, such as aggrecanase-1 and -2, MMP-3 and –13, responsible for cartilage destruction in osteoarthritis (OA) can be abrogated by exposure to n-3 polyunsaturated fatty acids (PUFAs). Similarly, the expression of inflammatory mediators such as cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX), interleukin-1 (IL-1) and TNFα were also down regulated by n-3 PUFA exposure.

The objective of this research was to extend these earlier studies to determine the proteomic expression of inflammatory mediators in the same experimental systems and to initiate new studies determining potential intracellular signalling pathways affected by n-3 PUFA metabolism. The specific aims to achieve this objective were:

1) To use chondrocyte monolayer and explant cultures stimulated with IL-1 as a model of cartilage degradation to investigate the effects of n-3 PUFAs on the protein expression and activity of inflammatory mediators, specifically COX-2.

2) To investigate the effects of n-3 PUFAs on chondrocyte intracellular signalling pathways and to identify possible target pathways for further investigation.

3) To specifically inhibit target signal pathways in order to investigate the effects on degradative enzymes and inflammatory mediators associated with cartilage destruction after supplementation with n-3 PUFA.

4) To perform a more detailed study on specific inhibition of a particular signalling pathway and to compare the results gained above with those obtained previously allowing possible target signal pathways effected by n-3 PUFAs to be predicted.
2.1 General Materials

Seven-day old bovine articular cartilage was obtained from the local abbatoir. Human osteoarthritic cartilage was obtained as waste from total knee replacement operations with Local Ethical Committee approval and patient consent from Llandough Hospital, South Wales, UK. Pronase (Streptomyces griseus) was from Boehringer Mannheim, UK. Collagenase II (Clostridium histolyticum) was from Worthington Biochemical Co., New Jersey, USA. Gentamicin and antibiotic/antimycotic (PSF) solution were both obtained from Invitrogen, Paisley, UK. Dulbecco’s Modified Eagle’s Medium (DMEM) and foetal calf serum (FCS) were from Gibco-BRL, Paisley, UK. All cell culture plates and filter units were obtained from Corning, UK, and Millipore UK, respectively. Recombinant human IL-1α and IL-1β were both purchased from Tebu-Bio, Peterborough, UK. All fatty acids are 99% purity and obtained from Sigma-Aldrich, Dorset, UK, as well as fatty acid free bovine serum albumin (Fraction V). PD98059, U0126 and SB203580 were from Alexis Biochemicals, UK, while curcumin and PDTC were also from Sigma-Aldrich. Lactate reagent and lactate standards were obtained from Trinity Biotech, Oxfordshire, UK. DMMB reagent and Chondroitin Sulphate C from shark cartilage used for the DMMB standards were both from Sigma-Aldrich. Acrylagel and bisacrylagel were obtained from National Diagnostics, UK. Pre-poured 4-12% gradient gels were from Invitrogen, UK. Precision plus protein standards were from Bio-Rad, Herts, UK. Chondroitinase ABC was from Sigma-Aldrich, Keratanase and Keratanase II were both from MP Biomedicals, London, UK. Monoclonal antibodies BC-3 and BC-14 were obtained from Prof. B. Caterson, Connective Tissue Biology Laboratories, Cardiff University, Wales, UK. Polyclonal antibodies specific for cyclooxygenase-2 were from Santa Cruz Biotechnology, USA, and a polyclonal antibody specific for p44/42 MAP Kinase (Thr202/Tyr204) was from Cell Signalling.
Technology, New England Biolabs, USA. Alkaline phosphatase (AP)-conjugated anti-goat and anti-rabbit secondary antibodies were from Sigma-Aldrich and AP-conjugate anti-mouse secondary antibody was from Promega, Southampton, UK. Tri-Reagent was obtained from Sigma-Aldrich. RNeasy miniprep kit for RNA extraction was obtained from Qiagen, Crawley, UK. Oligonucleotide primers were from Invitrogen. The RNA PCR kit [including PCR Buffer, MgCl₂, dNTPs, RNase Inhibitor, Reverse Transcriptase and oligo d(T)] was from Applied Biosystems, Warrington, UK. Taq polymerase (Thermus aquaticus) was from Promega. Agarose was from MP Biomedicals. Biomarker standards for RNA gels were from Cambio, Cambridge, UK.

2.2 Cartilage Isolation and Chondrocyte Cell Culture

2.2.1 Monolayer Cultures

Seven-day old bovine metacarpo- and metatarsophalangeal joints were obtained from the local abattoir and prepared. Full depth cartilage slices were cut away from the bone in sterile conditions and placed in DMEM (Gibco-BRL, Paisley, UK), containing 10% FCS (plus 50μg/ml gentamicin [Invitrogen, UK]), before being subjected to pronase (Streptomyces griseus) (0.1% in DMEM with 10% FCS for 3 hours) (Boehringer Mannheim, UK) and collagenase (Clostridium histolyticum) (0.04% in DMEM with 10% FCS overnight) (Worthington Biochemical Co., New Jersey, USA) digestion to isolate the chondrocytes (Hughes et al., 1998). Monolayer cultures were established in 60mm culture dishes by plating 1ml/dish of a suspension of about 6x10⁶ chondrocytes/ml of DMEM.

2.2.2 Explant Cultures

Explants were obtained from both bovine (as above) and human cartilage. Human cartilage was obtained as waste material from patients undergoing total knee replacement surgery for
osteoarthritis (Llandough Hospital, S.Wales, UK). All procedures had Local Ethical Committee Approval. The tissue was placed in physiological saline before further manipulation. Explant cultures were established by taking diced cartilage (20-70mg wet weight) and preculturing in petri dishes in 1ml of DMEM containing 10% FCS (plus 50µg/ml gentamicin, 0.5% antibiotic/antimycotic solution 100X concentrated [Invitrogen, UK] for the human cultures) for 72hrs at 37°C, 5% CO2, 95% air. Following this, explants were washed and DMEM added (minus FCS).

2.3 Lactate Assay

To evaluate the metabolic state of chondrocyte cultures a commercially-available lactate assay kit (Sigma-Aldrich, UK) was used to determine the concentration of lactate produced in the culture medium. This assay is based on the principle that lactic acid is converted to pyruvate by lactate oxidase and then, in the presence of hydrogen peroxide, peroxidase catalyses the oxidative condensation of chromogen precursors to produce a coloured dye with an absorption maximum at 540nm. Changes of lactate levels in the medium are thought to indicate changes in chondrocyte metabolism and, therefore, are indicative of cell viability. Lactate standards were prepared (0-400µg/ml). 5µl of standard or appropriately diluted media samples were added to individual wells of a 96 well plate. Lactate reagent (250µl) was then added to each well and the plate incubated at room temperature for 10mins before the absorbance was read at 540nm using a microplate reader (Labsystems Original Multiskan MS).

2.4 Dimethylmethylene Blue (DMMB) Assay

The DMBB assay was used to quantify the proteoglycan content in the chondrocyte culture medium by quantifying the levels of sulphated glycosaminoglycan (GAG) (Farndale et al., 1986). DMBB binds to the sulphate groups on GAGs forming a dye: GAG complex. The
formation of this complex produces a shift in the colour absorbance form blue to pink. Standards (chondroitin sulphate C isolated from shark cartilage, Sigma-Aldrich, UK) were made up with concentrations from 0-40μg/ml and placed in a 96 well plate together with the appropriately diluted media samples. 200μl of DMBB assay solution (Sigma-Aldrich, UK [32mg 1,9-DMBB, 20ml ethanol, 59ml 1M sodium hydroxide, 7ml 98% formic acid and made up to 2 litres with water]) was added to each well and the absorbance read immediately at 525nm.

2.5 SDS-PAGE and Western Blotting
Lyophilised samples were resuspended in 2X concentrate sample buffer (containing 0.125M Tris HCl, pH 6.8. 4% SDS, 20% glycerol, 0.01% bromophenol blue) and milli-Q water. The samples were reduced by adding β-mercaptoethanol at 10% of the final loading volume, and then heated for 10mins at 70°C prior to loading. The samples underwent electrophoresis in polyacrylamide gels (poured using acrylagel and bisacrylagel from National Diagnostics, UK) in SDS. After electrophoresis, the fractionated proteins were transferred electrophoretically to nitrocellulose membranes (Schleicher and Schuell, Germany), blocked with 5% bovine serum albumin/TSA for 1hr and immunobotted with a primary antibody chosen to be specific to a protein of interest, overnight. The primary antibody was poured off, the nitrocellulose membrane washed with TSA and a secondary antibody added to the membrane for 1hr. All antibodies were diluted with 1% BSA/TSA. The membrane was subjected to three 10min washes in TSA and developed in alkaline phosphatase substrate (10ml of buffer [100mM Tris, 100mM NaCl, 5mM MgCl₂, pH 9.55] with 66μl of nitroblue tetrazolium and 33μl of 5-bromo-4-chloro-1-indolyl phosphate) for 10-15 minutes or until optimum colour development of immunopositive bands was obtained.

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2.6 RNA Extraction and Isolation

2.6.1 Dismembrator Extraction of RNA

This protocol was applied to tissue obtained from explant cultures. The dismembrator shakes frozen tissue vigorously in the presence of a steel ball to break it up. The tissue was snap-frozen in liquid nitrogen along with Braun Mikro-Dismembrator vessels (B. Braun Biotech International GmbH, Germany). Tissue was quickly placed into the vessel with the ball and locked into the dismembrator. The tissue was then homogenised for 1 min at 2000rpm. The vessel was opened and Tri-Reagent (Sigma-Aldrich, UK) added (1 ml for samples up to 100 mg) to the powdered cartilage. After the Tri-Reagent and tissue had thawed, the sample was transferred to a 1.5 ml tube. This could then be stored at −20°C prior to RNA extraction using the protocol laid out in section 2.6.2, below (QIAGEN RNasea).

2.6.2 RNA Isolation on QIAGEN RNasea

The monolayer media from duplicate culture plates was pooled and if necessary frozen at −20°C for later analysis. To pulverised explant tissue or a cell layer in a 60 mm culture plate, 2 ml of Tri-Reagent (Sigma-Aldrich, UK) was added and left at room temperature for 10 min, allowing the cells to lift away from the plate. The cell/Tri-reagent suspension was then placed into a sterile screw-cap tube. A QIAGEN RNasea mini prep kit (Qiagen, Crawley, UK) was used to extract the RNA from Tri-Reagent (whether it be from monolayer or explant culture). To 2 ml of Tri-Reagent, 0.4 ml of CHCl₃ was added and the mixture left to sit at room temperature for 15 mins. This was then centrifuged at maximum rpm (13200 rpm) for 15 mins in a U-32R centrifuge (Boeco, Germany). The upper phase was transferred to a sterile 1.5 ml tube containing 375 μl of 70% ethanol and mixed by inverting. The mixture was applied to a spin column and RNA was isolated following the manufacturer's protocol. This extract could then be stored at −20°C until used in RT-PCR or array experiments. The RNasea method isolates all RNA molecules of 200

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nucleotides or more. The conditions used will not allow small RNAs to bind to the membrane and, hence, the extraction enriches for longer RNA molecules. Absorbance ratios were measured at 260/280nm and RNA concentrations (µg/ml) calculated using an Ultraspec 2000 (Pharmacia Biotech, UK).

2.7 Oligonucleotide Primer Design

Before constructing cDNA oligonucleotides certain design criteria had to be considered. Each primer should:

- be between 20 and 25 nucleotides long
- have annealing temps between 50-65°C
- have a GC content of 40-60%
- avoid being self-complimentary to reduce primer-dimer formation

Human and bovine gene sequences of interest were found using Genbank and the primer pairs designed using the Oligo 4.0s program for Macintosh. The primer pairs used are described in Table 2.1.

2.8 Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

RT-PCR methods were utilised to examine transcription patterns of various signalling genes and the gene expression of matrix proteins. Reverse transcriptase–PCR was performed using the RNA PCR kit (Applied Biosystems, Warrington, UK) consisting of Gene Amp 10X PCR buffer II (100mM Tris-HCl pH 8.3, 500mM KCl), MgCl₂ (25mM solution), dNTPs (dATP, dCTP, dGTP, dTTP, all at 10mM), RNase Inhibitor, MuLV Reverse Transcriptase and oligo d(T). First strand cDNA was synthesised by reverse transcription using MuLV reverse transcriptase and PCR amplification was performed using oligonucleotide primers (Invitrogen, UK) corresponding
<table>
<thead>
<tr>
<th>Target</th>
<th>PCR Primer Sequences (5'-3')</th>
<th>Annealing Temp (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>TGGCATCGTGGAAGGGGCTCAT</td>
<td>50.0</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>ATGGGAGGCTGGTCTGTTGAAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine COX-2</td>
<td>GCTCTTTCCTCCTGCTGCTGAT</td>
<td>52.3</td>
<td>229</td>
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<tr>
<td></td>
<td>CATGGTTCTTTCCCTTAGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human COX-2</td>
<td>GGCTGTCCCTTACTTCATT</td>
<td>48.2</td>
<td>438</td>
</tr>
<tr>
<td></td>
<td>ACATCTTTACTTCGCCCTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine/Human MMP-3</td>
<td>CTTTTGGCGAAAAATCTCTCAG</td>
<td>50.0</td>
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<td></td>
<td>AAAGAAACCCAAATGCTTCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine/Human MMP-13</td>
<td>TTCTGGCACCACGCTTTTCTTC</td>
<td>53.0</td>
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<tr>
<td></td>
<td>GGTGGGGTCTTCATCTCCTG</td>
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</tr>
<tr>
<td>Bovine/Human TIMP-1</td>
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<tr>
<td></td>
<td>CCTCACAGCCACACAGTGAGG</td>
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</tr>
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<td>Bovine/Human TIMP-2</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>GCCGGATGCAGCGGCTGTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine/Human ADAMTS-4</td>
<td>TGGATCCCGAGGAGCCTGTTG</td>
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<td>151</td>
</tr>
<tr>
<td></td>
<td>TGGCGGTGCAGCGTGTAGTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine/Human ADAMTS-5</td>
<td>GGCTCTCTCCATGAYGATTCC</td>
<td>59.2</td>
<td>498</td>
</tr>
<tr>
<td></td>
<td>TGAGCGAGAACACTGGCCCCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine/Human Aggrecan</td>
<td>CGCTACGACGCCATCTGCTAC</td>
<td>57.0</td>
<td>497</td>
</tr>
<tr>
<td></td>
<td>GCCTGCTGTGCTCCTCACA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1** Oligonucleotide primers used in RT-PCR (purchased from Invitrogen, UK). Sequences, annealing temperatures and product sizes are shown. In one case a letter is used to indicate a mixed base pair (e.g. Y = C and T).
to cDNA sequences from various degradative enzymes and inflammatory factors (see Table 2.1). Following an initial denaturation step of 30 sec at 95°C, amplification consisted of 30-45 cycles of 30 sec at 95°C, 45 sec at the individual annealing temperature of the primers, 45 sec at 72°C, followed by a final extension step of 5 min at 72°C. The PCR products were visualised on 3% agarose gels (containing 2 μl ethidium bromide per 30 ml of gel) under UV light (Flannery et al., 1999).

When a single product band was not seen, a hot start PCR technique was used to optimise the PCR conditions. This method prevents complete mixing of the PCR reactants until the reaction reaches a temperature at which non-specific annealing of primers to non-target DNA is minimised and primer oligomerisation rate reduced. A solid wax layer (Ampliwaq PCR gem wax beads, Perkin Elmer, UK) forms over a subset of PCR reactants (10X PCR Buffer II, MgCl₂, dNTPs, primers) and the remaining reactants (10X PCR buffer II, Taq polymerase [from Thermus aquaticus strain YT1, Promega, UK], and RT-sample) were added on top. In the first thermal cycle rapid heating to the denaturation temperature (95°C) melts the wax and the reagents can mix.

2.9 Purification of PCR Products and Verification of Nucleotide Sequences

Wizard PCR Preps DNA Purification System (Promega, UK) was used to purify the PCR products following the manufacturer’s guidelines. The PCR products were then sequenced to check template specificity of each primer by using an Applied Biosystems ABI310 Genetic Analyser. This was followed by the alignment of nucleotide sequences using the MacDNASIS sequence analysis software package (Hitachi Software, San Francisco, USA).
CHAPTER 3 - THE EFFECTS OF n-3 POLYUNSATURATED FATTY ACIDS ON COX-2 PROTEIN LEVELS AND ENZYME ACTIVITY IN CARTILAGE METABOLISM

Cyclooxygenase-2 (COX-2) induction has been observed in both human osteoarthritic (OA) cartilage (Amin et al., 1997; Curtis et al., 2002) and in synovial tissue taken from patients affected with rheumatoid arthritis (RA) (Kang et al., 1996). In vitro cell culture experiments have also shown that inflammatory agents IL-1, TNF-α and LPS, as well as the growth factors TGF-β, EGF, PDGF and FGF, will induce COX-2 expression (Crofford, 1997).

Non-steroidal anti-inflammatory drugs (NSAIDs e.g. aspirin and ibuprofen) are capable of controlling prostaglandin-induced inflammatory responses via inhibition of prostaglandin synthesis by COXs. This inhibition blocks inflammation but is also associated with a breakdown in the normal tissue homeostasis by controlled COX activity. For example, gastric mucosal protection is impaired and can lead to adverse gastrointestinal effects in some patients. Such problems have mainly been attributed to COX-1 inhibition by NSAIDs and, because of this, selective COX-2 inhibitors, such as Rofecoxib and Valdecoxib (Chavez and DeKorte, 2003; Crofford, 2002), have been designed which retain their anti-inflammatory properties without affecting any of the house-keeping roles of COX-1 (Cryer and Dubois, 1998).

Previous work within our laboratory (Curtis et al., 2000; 2002) has shown that n-3 polyunsaturated fatty acids (PUFAs), such as those found in fish oils, but not other classes of fatty acids have a beneficial effect on reducing the mRNA levels and activity of degradative and inflammatory factors known to cause damage and destruction in arthritic diseases. On supplementation of n-3 PUFAs, there is a dose-dependent reduction in the mRNA and protein levels of proteoglycan degrading enzymes (aggrecanases) and the mRNA levels of inflammation-inducible cytokines (IL-1α, TNF-α) as well as enzymes that produce inflammatory prostaglandins and leukotrienes (COX-2, 5-LOX and FLAP). Because changes in
mRNA do not, necessarily, cause changes in cellular activity, my aim was to investigate the effects that the same PUFAs had on the synthesis of inflammatory proteins and enzyme activity, specifically of cyclooxygenase-2 (COX-2) in the same experimental systems.

3.1 Materials and Methods

Articular cartilage was obtained from both 7-day old bovine metacarpo-metatarsophalangeal joints and from human patients (obtained as surgical waste from patients undergoing total knee replacement surgery for OA, using procedures approved by the Hospital Institutional Review Board). Both monolayer and explant cultures were established as described in sections 2.2.1 and 2.2.2.

3.1.1 Polyunsaturated Fatty Acid-Treated Monolayer Cultures

Bovine monolayer cultures were maintained overnight (at least 8hr) in the absence or presence of 10–100 μg/ml n-3 PUFAs (α18:3 linolenic acid, 20:5 eicosapentaenoic acid) or the n-6 PUFAs (18:2 linoleic acid, 20:4 arachidonic acid) (see Table 3.1). These concentrations are the equivalent to the ranges achievable in various mammalian plasmas (Takita et al., 1996). All fatty acids were a minimum of 99% purity, obtained from Sigma-Aldrich (Poole, UK). Prior to their addition to cultures, all PUFAs were incubated for 16hrs at 37°C in Tyrode-HEPES buffer (20mM HEPES, 140mM NaCl, 4.5mM KCl, 1mM MgCl₂, 2.5mM CaCl₂, 11mM glucose, pH 7.4) with 3.5mg/ml fatty acid-free bovine serum albumin (Fraction V) (Sigma-Aldrich). After overnight incubation with fatty acids, the culture medium was removed, the cells washed with fresh DMEM and new media (without fatty acids) was added with or without 10ng/ml IL-1α (Tebu-Bio, UK). The cells were then further incubated at 37°C for a period of up to 4 days before harvesting (Curtis et al, 2000).
<table>
<thead>
<tr>
<th>Fatty Acid Class</th>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-3 (omega-3)</td>
<td>α18:3 Linolenic acid</td>
<td>CH$_3$CH$_2$(CH=CHCH$_2$)$_7$(CH$_2$)$_9$COOH</td>
</tr>
<tr>
<td></td>
<td>20:5 Eicosapentaenoic acid</td>
<td>CH$_3$CH$_2$(CH=CHCH$_2$)$_7$(CH$_2$)$_9$COOH</td>
</tr>
<tr>
<td>n-6 (omega-6)</td>
<td>18:2 Linoleic acid</td>
<td>CH$_3$(CH$_2$)$_2$CH=CHCH$_3$CH=CH(CH$_2$)$_7$COOH</td>
</tr>
<tr>
<td></td>
<td>20:4 Arachidonic acid</td>
<td>CH$_3$(CH$_2$)$_4$(CH$_2$CH=CH)$_4$(CH$_2$)$_9$COOH</td>
</tr>
</tbody>
</table>

**Table 3.1** Fatty acids supplemented in the culture medium. All fatty acids were obtained from Sigma-Aldrich, UK.

3.1.2 Polyunsaturated Fatty Acid-Treated Explant Cultures

Bovine and human pre-cultured explants were washed (3 x 10min) in serum-free DMEM and maintained for 24hrs in individual wells of 24-well plates with 1ml of serum-free DMEM with or without 10-100μg/ml (bovine explants) or 100-300μg/ml (human explants) n-3 or n-6 PUFAs complexed to fatty acid-free BSA. After this period of culture the medium was removed and the explants washed with DMEM and fresh medium (no PUFA supplementation) applied with or without 10ng/ml IL-1 (IL-1α for bovine explants and IL-1β for human). Explants were then cultured for 4 days under the same conditions before being harvested.

3.1.3 Lactate Assay and DMMB Assay Analyses

The lactate assay (designed by Sigma-Aldrich, UK) was used to evaluate the metabolic state of all culture media following the protocol described in section 2.3 and to ensure the PUFAs were not having a detrimental effect on general tissue metabolism. A significant decrease in lactate production during treatment was interpreted as a detrimental change in cell viability or metabolism.
DMMB assays (see section 2.4) were also carried out on all media collected to ensure the PUFAs were having the same effects as previously seen in other research. Thus with an increasing dose of n-3 PUFA, a decrease in extracellular GAG was expected in the explant culture media, with no change seen following n-6 PUFA supplementation (Curtis et al., 2000; 2002).

3.1.4 RT-PCR using Oligonucleotides Specific for COX Expression

RNA was extracted from bovine and human explants as described in section 2.6 and oligonucleotide primers were designed to specifically recognise COX-2 in either bovine or human articular cartilage. RT-PCR and sequence analysis was carried out as described in sections 2.8 and 2.9. Primer pair sequences and RT-PCR conditions are outlined in Table 2.1.

3.1.5 Cyclooxygenase Protein Extraction Protocol

Monolayer chondrocytes were washed twice with cold PBS and, using a rubber policeman, were scraped from their plates into 1ml of cold lysis buffer (40mM Tris, 1mM EDTA, 250mM sucrose, 5mM DTT, 10mM NaF, 0.2mM Na3VO4, 0.5mM PMSF, 10µg/ml leupeptin, 1µg/ml antipain, 1µg/ml pepstatin A), placed into tubes and kept on ice. The suspension was then subjected to homogenisation by 30 passages through a narrow gauge needle and syringe. The efficiency of homogenisation was checked under a microscope. The homogenate was then centrifuged at 13,000rpm for 10mins at 4°C and the supernatant kept as the protein source (Thomas et al., 2002). The absorbance of each sample was read at 280nm using an Ultraspec 2000 (Pharmacia Biotechnologies, UK) to check that protein extraction had been successful. The samples were then dialysed overnight in water and lyophilised; the latter were stable to store at room temperature until later use.
3.1.6 Analysis of Cyclooxygenase Presence in Cartilage Monolayer Cultures Using SDS-PAGE and Western Blotting Techniques

Reduced samples were electrophoresed on 10% polyacrylamide gels in SDS following the standard protocol outlined in section 2.5, transferred to nitrocellulose membranes and blocked with 5% BSA/TSA. The membranes were immunoblotted with the polyclonal primary antibodies; goat anti-COX-1 and goat anti-COX-2 (Santa Cruz Biotechnology, USA), overnight. Both primary antibodies were used at 1:1000 diluted with 1% BSA in TSA. The primary antibody was removed, the membranes washed with TSA and an alkaline-phosphatase conjugated anti-goat secondary (Sigma Aldrich, UK), was added to the membrane at a concentration of 1:10,000 in 1% BSA, for 1 hour. The membrane was washed, substrate added and developed as previously described (see section 2.5).

3.1.7 PGE₂ ELISA Immunoassay

The PGE₂ immunoassay used a commercially available kit (R&D Systems, UK) based on the competitive binding technique in which the PGE₂ present in a sample competes with a fixed amount of alkaline phosphatase-labelled PGE₂ for sites on a mouse monoclonal antibody. During the incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. The plate is washed to remove excess conjugate and unbound sample and a substrate solution added to determine the bound enzymes activity. This is seen visually as a change in colour, inversely proportional to the concentration of PGE₂ in the sample.

Culture media from cartilage explant cultures was analysed and fresh DMEM was used for the controls and as a diluting agent. The experiment was carried out in a 96 well microplate with wells reserved for both the total activity (TA) and substrate blank reactions. Fresh DMEM (150µl) was added to wells reserved for the non-specific binding (NSB) reaction and also to the
maximum binding (B_{max}) wells (100\mu l). PGE_2 standards were made ranging between 39–5000pg/ml and added to individual wells (100\mu l).

Sample media to be analysed were added to the remaining wells (100\mu l). All reactions were carried out in duplicate. PGE_2 conjugate (50\mu l) was added to each well (excluding the TA and substrate blank wells) and PGE_2 antibody solution (50\mu l) added to each well, except the NSB, TA and substrate blank. The plate was then covered and allowed to incubate for 2 hours, at room temperature on an orbital microplate shaker. After the incubation, the wells were aspirated and washed a total of three times with wash buffer (200\mu l). PGE_2 conjugate (5\mu l) was then added to the TA wells only and pNPP substrate (200\mu l) added to all wells. The plate was then incubated at room temperature on the bench top. After 2 hours the optical density was determined using a microplate reader (Labsystems Original Multiskan MS) set at 405nm. All optical densities (OD) were corrected by subtracting the non-specific binding (NSB) OD from the readings taken at 405nm. A standard curve was then generated by plotting the corrected mean absorbance for each standard on a linear y-axis against its concentration on a logarithmic x-axis. The standard curve could then be used to calculate the concentration of PGE_2 within a sample.

3.1.8 Statistical and Densitometrical Analysis

RT-PCR and Western band quantification was carried out using NIH Image and Statview 4.02 software packages for Mac. PCR gels were photographed under UV light. The photos or nitrocellulose membranes were scanned using an Epson Expression 1680 Pro scanner. By importing the pictures into the NIH Image program, densometric analysis could be carried out. Statview was used to generate bar graphs to illustrate changes in expression levels. For PCR, the housekeeping gene, GAPDH was used for normalisation.

PGE_2 analysis involved using Microsoft Excel (Office 2001) and the Statview 4.02 software package for Mac. Standard curves were generated using Excel and once PGE_2 concentrations
were calculated, significance testing was carried out using ANOVA (analysis of variance). The ANOVA test is a way of testing hypotheses about differences between two or more sample means. The t-test is only reliable when testing two means only and multiple t-tests on more than two means can result in a high rate of error. Thus, ANOVA can be used to test differences among several means for significance without increasing the rate of error. This test is used to uncover the effects of independent variables on an interval dependent variable (e.g. in this case, the effects of n-3 PUFA dosage on PGE₂ production). A confidence interval (or p value) is then applied to evaluate how reliable the change is. The conventional threshold in science to conclude if the evidence is strong enough, or not, is 0.05 and this was used for this analysis (Grafen and Hails, 2002).
3.2 Results

3.2.1 Analysis of Cell Viability after PUFA Supplementation

The lactate assay was used as a means of testing cell viability, as previously described in sections 2.3 and 3.1.3. Media collected from bovine monolayer (Figure 3.1A), explant (Figure 3.1B) and human OA explant (Figure 3.1C) cultures were analysed.

The bovine monolayer culture results (Figure 3.1A), showed an increase in lactate production with the addition of IL-1 compared to the control cultures, indicating an increase in non-aerobic cell metabolism on addition of this cytokine. There was no statistically significant change in lactate release with PUFA supplementation compared to either control or IL-1 induced cultures. Hence PUFA supplementation had no detectable effect on lactate production as a measure of general cell metabolism.

In the bovine explant cultures (Figure 3.1B) a significant PUFA effect was seen after treatment with 10µg/ml EPA compared to the control cultures although this decrease was not found with higher concentrations of EPA and did not cause a change in cell numbers. No other treatments showed any statistically significant change in lactate production compared to the controls and PUFA supplementation has no effect on lactate production in IL-1 treated explants.

There was also no statistically significant change in lactate production in any of the human OA explant media samples compared to the controls and, hence, there was no effect on cell viability after IL-1 or PUFA supplementation (Figure 3.1C).
Figure 3.1 Lactate analysis of medium from A) bovine monolayer B) bovine explant and C) human explant cultures. Cultures were supplemented with or without 10-300\(\mu\)g/ml \(n\)-3 PUFA (EPA) or \(n\)-6 PUFA (AA) for 24 hours followed by plus or minus 10ng/ml IL-1 for 4 days. (Means ± S.D.; n=12). Data from 3-4 experiments, carried out in triplicate are shown. * = significant increase versus control (p<0.003) (ANOVA test).

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3.2.2 GAG Release from PUFA Supplemented and IL-1 Stimulated Chondrocytes

Previous work has shown that n-3 PUFAs, but not n-6 PUFAs, have a beneficial effect on reducing the levels of GAG released (following connective tissue catabolism) into the culture medium from IL-1 treated cultures (Curtis et al., 2000; 2002). The DMMB assay was used as a means of testing GAG release and, hence, the PUFA effects on chondrocyte metabolism, as previously described in sections 2.3 and 3.1.3. Media collected from bovine monolayer (Figure 3.2A), explant (Figure 3.2B) and human OA explant (Figure 3.2C) cultures were analysed.

Addition of IL-1 to bovine monolayer cultures appeared to result in a decrease in GAG release into the medium (Figure 3.2A). In monolayer cultures, media GAG alone is not the best measure of degradation as it does not take into account the large decrease in proteoglycan synthesis observed on the addition of IL-1. Therefore, a better measure would have been to measure the release of GAG expressed as a percentage of the total (Tyler, 1985). Hence, it is difficult to interpret the changes in media GAG in the monolayer culture system. In bovine explant culture medium GAG release is significantly increased (up to 5 fold) compared to control cultures when stimulated with IL-1α (Figure 3.2B). The addition of 10μg/ml EPA had no significant effect, but the addition of 50μg/ml EPA reduced GAG release significantly, whilst 100μg/ml further lowered it back down to basal levels. In contrast, the addition of AA (at 10μg/ml) to IL-1 stimulated bovine explants significantly increased the GAG release even further. However, this effect of AA was not found at higher concentrations where GAG release was the same as IL-1 treated controls. It has previously been shown that IL-1 stimulation significantly increases GAG release from bovine explants and that n-3 PUFAs, but not n-6 PUFAs, have a dose-dependent effect on decreasing this IL-1 induced GAG release (Curtis et al., 2000). Therefore, the data in Figure 3.2 are in general agreement with this report.

Human OA explant cultures showed a small but significant increase in GAG release compared to control cultures when stimulated with IL-1 (Figure 3.2C). However, it should be noted that this
Figure 3.2 GAG analysis of medium from A) bovine monolayer B) bovine explant and C) human explant cultures, supplemented with or without 10-300μg/ml n-3 PUFA (EPA) or n-6 PUFA (AA) for 24 hours, plus or minus 10ng/ml IL-1 for 4 days. (Means ± S.D.; n≤12). Data from 3-4 experiments, carried out in triplicate are shown. Significant changes tested using ANOVA.

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reduction in GAG release was not to the same levels seen in bovine explants. Human articular
cartilage tissue was obtained from OA knee replacement operations and is, hence, highly
pathological already so the addition of IL-1 does not necessarily induce further GAG release. In
this experiment, addition of n-3 PUFAs did not result in a significant decrease of GAG release.
However, due to the very low levels of GAG in these pathological tissue samples, it was difficult
to achieve a significance value.

3.2.3 The Effects of n-3 PUFAs on Cyclooxygenase-2 mRNA Expression

Oligonucleotide primers were designed for use in RT-PCR and were specific to either the human
or the bovine forms of cyclooxygenase-2. Total RNA was isolated from explant tissue. It can be
seen that bovine COX-2 mRNA expression is induced by exposure to IL-1α compared to control
cultures (Figure 3.3A). Analysis of the human osteoarthritic cartilage showed that COX-2
message was already present in the ‘control’ human samples (Patient 1 and 3, Figure 3.4), which
is indicative of the excessive inflammatory response in the pathological joint tissues. The human
COX-2 mRNA levels were further elevated upon treatment with IL-1β. After supplementation
with EPA at a concentration of 100μg/ml (bovine) or 300μg/ml (human) there was a marked
reduction in mRNA levels (Figure 3.3A and Patients 1 and 2, Figure 3.4). However, this was not
seen when IL-1 induced samples were treated with the n-6 PUFA (AA) (Figures 3.3A and 3.4)
(see histograms of densitometrical scans shown in figures 3.3B and 3.4B, respectively). Data
from the bovine explant cultures (Figure 3.3) are similar to those published by Curtis et al.,
(2000) and those repeated by other members of the Caterson and Harwood Laboratories (results
not published). Data for the positive effects of n-3 PUFA supplementation to cartilage from
patients with osteoarthritis was variable between patients. Of the three patients analysed, two
appeared to show positive effects (Patients 1 and 2, Figure 3.4). These data are similar to that
described by Curtis et al., (2002).
Figure 3.3 mRNA expression of COX-2 in 7-day old bovine articular cartilage. RNA was isolated from explants supplemented with or without 100μg/ml n-3 PUFA (EPA) or n-6 PUFA (AA) for 24 hours, washed and followed by exposure to 10ng/ml IL-1α for 4 days. A) RT-PCR carried out using oligonucleotide primers (Invitrogen, UK) specific to bovine COX-2. B) Quantification was carried out using NIH Image and Statview 4.02 software packages, represented here in histogram form. C) RT-PCR of the housekeeping gene GAPDH. One representative experiment is shown here.
Figure 3.4 mRNA expression of COX-2 in human osteoarthritic tissue from three female patients undergoing total knee replacement surgery (aged 61, 75 and 79 years). Explant cultures were supplemented with or without 300µg/ml n-3 PUFA (EPA) or n-6 PUFA (AA) for 24 hours, washed and followed by exposure to 10ng/ml IL-1β (human cultures) for 4 days. A) RT-PCR was carried out using oligonucleotide primers (Invitrogen, UK) specific to human COX-2 and to the house-keeping gene, GAPDH. B) Quantification was carried out and COX-2 expression levels standardised to GAPDH using NIH Image and Statview 4.02 software packages. Further analysis on other human knee OA patients has given similar results.
3.2.4 The Effects of n-3 PUFAs on Cyclooxygenase-2 Protein Levels

SDS-PAGE and Western Blot analysis was carried out on bovine chondrocytes grown in monolayer cultures using antibodies specific to the 72kDa COX-2 protein (Figure 3.5) in order to compare protein levels with changes in mRNA levels. Bovine COX-2 protein levels were increased after IL-1α (Figure 3.5, lane 4) exposure compared to the control cultures (Figure 3.5, lane 1). Supplementation with EPA (100μg/ml) had the effect of greatly reducing COX-2 protein levels in control and IL-1-treated cultures (lanes 2 and 5, respectively) back to or below that of the control levels (lane 1). Supplementation with AA caused the highest levels of COX-2 protein in both control and IL-1 treated bovine cultures and there appears to be triple bands present on the blot (Figure 3.5, lanes 3 and 6). This experiment was repeated several times with similar results each time and was also performed with other n-3 and n-6 PUFAs namely α18:3 (linolenic) and 18:2 (linoleic) acids, respectively, again with similar results (data not shown).

Similar experiments examining COX-2 protein levels in cells from human OA chondrocyte monolayer cultures were attempted several times. Problems were encountered due to the low numbers of viable cells obtained from the tissue to be used in monolayer culture. Very low cell yields were obtained after digestion with pronase and collagenase due to the pathological state of the tissue. By passaging the cells up to four times, larger cell numbers were obtained. Protein extraction, SDS PAGE and Western were carried out but with very varied results. It was noted, however, that work by Hardingham’s group has shown that passaging chondrocytes can have an effect on cell phenotype. They have shown that, as the cell numbers expand, the chondrocytes lose their characteristic spherical morphology and instead spread, begin to proliferate and acquire a fibroblastic appearance. The group has also found that there is a decrease in collagen type II and aggrecan gene expression and a decline in the chondrogenic transcription factor SOX9 with an accompanying up regulation of collagen type I expression (Li et al., 2004). So it is possible
Figure 3.5 Western blot analysis of COX-2 protein expression to show the effects of 100μg/ml n-3 PUFA (EPA) or n-6 PUFA (AA) on bovine articular cartilage chondrocyte metabolism with or without 10ng/ml IL-1α.
that, after passaging human chondrocytes that the expression of COX-2 is altered. Further work
would need to be carried out in this area to ascertain if this is indeed the case.

3.2.5 The Effects of n-3 PUFA s on COX-2 Activity and PGE₂ Production

Using a commercially available PGE₂ ELISA immunoassay kit it was possible to analyse the
PGE₂ levels present in explant media from PUFA supplemented bovine and human samples.
PGE₂ standards were prepared and standard curves generated (see protocol, sections 3.1.7 and
3.1.8) using a 2-hour incubation period at room temperature. The reproducibility of these
standard curves from 9 assays over a six-month period is shown in Figure 3.6. Articular cartilage
explant cultures were supplemented with or without n-3 or n-6 PUFA s (EPA or AA) and, then,
in the presence or absence of IL-1. Culture media from bovine explant cultures were analysed for
PGE₂ levels (Figure 3.7). The concentrations of bovine PGE₂ protein released into the
surrounding media were very low (near zero pg/ml) for control samples. However with IL-1α
treatment, this increased by over 2000-fold. This induced PGE₂ concentration was significantly
reduced with increasing doses of n-3 PUFA (10-100µg/ml) in a dose-dependent manner (Figure
3.7). Supplementation with AA had no effect on PGE₂ release in bovine media (data not shown).
The reduction in PGE₂ release by EPA was in agreement with the lowered levels of COX-2
protein (Figure 3.5).

Articular cartilage explant culture media from the knees of human OA patients was also analysed
(Figure 3.8). Explant media from all control samples showed low PGE₂ concentrations that were
greatly increased with the addition of IL-1β. Four patients were analysed; three females aged 73,
80 and 81 years and one male aged 69 years. Each patient reacted in the same manner to
supplementation with the n-3 PUFA, EPA, and showed significantly decreased protein levels
(back down at least to basal levels), but with no benefit of n-6 PUFA supplementation (Figure
3.8).
**Figure 3.6** Standard curves were generated for the PGE₂ ELISA Immunoassay (R & D System, UK) after incubation with substrate for 2 hours. Optical densities (OD) were read at 405nm on a Labsystems Original Multiskan MS plate reader and corrected by subtracting the non-specific binding OD. Microsoft Excel Software was then used to generate standard curves of absorbance versus PGE₂ concentration. PGE₂ concentrations in culture medium could then be calculated. The reproducibility of nine different ELISA analyses over a six month period are shown.
Figure 3.7 PGE$_2$ production in bovine explant cultures supplemented with or without 10-100µg/ml eicosapentaenoic acid. Cultures were supplemented for 24 hours, washed and then exposed to 10ng/ml IL-1α for 4 days. PGE$_2$ release into the culture medium was analysed using an ELISA Immunoassay (R&D Systems, UK) following the manufacturers protocol. (Mean ± S. D., n=4) * = p<0.0001 versus IL-1, ** = p<0.0001 versus IL-1 + 50µg/ml EPA.
Figure 3.8 PGE$_2$ production in human OA explant cultures supplemented with or without 300µg/ml n-3 eicosapentaenoic acid or n-6 arachidonic acid. Cultures were supplemented for 24 hours, washed and followed by exposure to 10ng/ml IL-1β for 4 days. PGE$_2$ release into the culture medium was analysed using an ELISA Immunoassay (R&D Systems, UK) following the manufacturer's protocol. [Mean ± S. D., n=4 individual patients (3 females aged 73, 80 and 81 plus one 69 year old male, triplicate samples were taken from each patient)], *= p<0.05 versus control, **= p<0.05 versus IL-1 alone.
3.3 Discussion

Collectively these data indicate that IL-1 induced the increase of COX-2 mRNA, the translated protein and its activity as measured by PGE₂ production in both monolayer and explant culture systems. These increases could be abrogated by supplementation with the n-3 PUFAs, eicosapentaenoic acid and α-linolenic acid, but not the n-6 PUFAs, arachidonic acid and linoleic acid. Previous work has shown that EPA competes with AA to become incorporated into cell membrane phospholipids. This leads to a decrease in the amount of AA available for eicosanoid synthesis (see Figure 3.9 and for a recent review see Calder, 2003b). Indeed, EPA has been shown to inhibit oxidation of AA by cyclooxygenase in cultured human mast cells and fish oil containing EPA was shown to decrease the production of prostaglandins of the 2-series e.g. PGE₂, thromboxanes like TXA₂ and also leukotrienes of the 4-series e.g. LTB₄ (Obata et al., 1999). Research data presented in this chapter suggests that EPA is having a similar effect on eicosanoid synthesis in bovine and human articular cartilage chondrocyte culture systems. Further work would need to be carried out to elucidate whether the thromboxanes are being effected in the same manner. However, it has been published previously from our laboratory that IL-1 induced 5-lipoxygenase and its activating protein, FLAP, mRNA expression is reduced in the presence of n-3 PUFAs but not n-6 PUFAs (Curtis et al., 2002). Hence, it is possible that LTB₄ production is also abrogated.

It is also known that EPA is able to act as a substrate for COX and give rise to products of a different nature and chemical structure to those produced when AA is the substrate. Again, further investigation would confirm whether 3-series prostaglandins and 5-series leukotrienes increase in concentration whilst the AA generated eicosanoids decrease, after supplementation with EPA. It is thought that the above eicosanoids, produced via the COX and LOX pathways using EPA as a substrate, are less inflammatory than those derived from AA.

My data suggests that n-3 PUFAs such as EPA found in oily fish and fish oils such as cod liver
Figure 3.9 Schematic to emphasise the anti-inflammatory effects of eicosapentaenoic acid (EPA) compared to arachidonic acid (AA). Abbreviations: COX, cyclooxygenase; LOX, lipoxygenase; LT, leukotrienes; PG, prostaglandin; TX, thromboxanes; PLA₂, phospholipase A₂ (Calder, 2003b).
oil could potentially relieve symptoms of inflammation caused by increased production of COX-2 and, hence, PGE₂ in osteoarthritis and other inflammatory diseases. By lowering COX-2 activity it is possible that n-3 PUFAs could reduce many of the pro-inflammatory effects known to be caused by the presence of PGE₂, TXA₂ and LTB₄, such as fever, pain, vasodilation leading to enhanced local blood flow, enhanced vascular permeability, platelet aggregation, leukocyte chemotaxis and adhesion, smooth muscle cell contraction and increased production of pro-inflammatory cytokines (Calder, 2003b). All these factors increase discomfort, swelling and pain in an inflamed joint.

3.4 Chapter Summary

- Neither IL-1 treatment nor PUFA supplementation have a significant effect on lactate release and, hence, cell viability and general metabolism in bovine and human articular cartilage explant cultures.
- IL-1 induced GAG release from bovine articular cartilage explant cultures is abrogated by supplementation with n-3 PUFAs but not n-6 PUFAs.
- IL-1 induced COX-2 mRNA expression is reduced back to or below basal levels after supplementation with n-3 PUFAs in both bovine and human articular cartilage explants, but not with n-6 PUFAs.
- Bovine COX-2 protein expression is induced by IL-1α treatment and is reduced by the supplementation of n-3 PUFAs but not n-6 PUFAs.
- COX-2 activity and, hence, production of PGE₂ into the surrounding explant media is increased by IL-1 in both bovine and human systems. The increased PGE₂ production is abrogated by the addition of n-3 PUFAs but not n-6 PUFAs.
CHAPTER 4 - THE EFFECTS OF \textit{n-3} POLYUNSATURATED FATTY ACIDS ON
INTRACELLULAR SIGNALLING PATHWAYS IN CARTILAGE METABOLISM

The beneficial effects that \textit{n-3} PUFAs have on reducing the expression and activity of
degradative and inflammatory factors known to cause cartilage damage and destruction in
arthritic diseases are well documented although their modes of action are still largely unknown
(Curtis et al., 2000; 2002). However, we have yet to show whether \textit{n-3} polyunsaturated fatty
acids are affecting gene transcription directly, whether fatty acid metabolites are affecting
downstream pathways that can alter gene transcription and/or whether it is mRNA stability that
is affected.

Until recent times, the analysis of gene regulation and function has largely been driven by step-
by-step studies of individual genes and proteins. However, it is now possible to produce large
amounts of data about many genes due to the development of DNA microarrays. These arrays
consist of a highly ordered matrix of up to tens of thousands of different DNA sequences that can
be used to measure DNA and RNA variation in applications that include gene expression
profiling, comparative genomics and genotyping (Harrington et al., 2000). Each array consists of
a reproducible pattern of different DNAs and provides a way of analysing many genes at a single
time. The PathwayFinder-1 GEArray kit (Cambridge Bioscience, UK) uses a very simplified
array system to investigate the RNA expression patterns of a variety of genes from several
signalling pathways. This then enables specific pathways to be identified for further investigation
using other methods.

In inflammatory disease such as arthritis, expression patterns of diverse cell types contribute to
the pathology. Joint inflammation is caused by the gene products of many cell types present in
synovium and cartilage tissues as well as those infiltrating from the circulating blood. Within
these cell types, mRNA from chondrocytes provides expression profiles for selected cytokines,
chemokines, DNA binding proteins and matrix degrading metalloproteinases (Heller et al., 1997). Such analyses may therefore, provide specific information to apply to joint inflammation and can supplement data from more general RNA preparations. Therefore, the same arrays can be used to analyse RNAs isolated from both specific cells as well as complex tissues.

The specific aims for this chapter were to use PathwayFinder-I GEArrays to investigate the effects of n-3 PUFAs on human chondrocyte intracellular pathways and to identify possible target pathways for further investigation.

4.1 Materials and Methods
Articular cartilage was obtained as surgical waste from human patients undergoing total knee replacement surgery for OA (Llandough Hospital, South Wales), with local ethical approval and informed patient consent.

4.1.1 Polyunsaturated Fatty Acid-Treated Explant Cultures
Human osteoarthritic articular cartilage explants were cultured as previously described in section 3.1.2 and supplemented with or without n-3 PUFA or n-6 PUFA (300µg/ml EPA or AA, respectively), overnight and in the absence or presence of IL-1β (10ng/ml), for a further four days.

4.1.2 Lactate Assay and DMMB Assay Analyses
The lactate assay (section 2.3) and the DMMB assay (section 2.4) were used to analyse the cartilage explant’s metabolic state as well as the GAG release from the tissue to the surrounding media, respectively.
4.1.3 Pathway Specific Gene Expression using a Microarray

SuperArray GEArray Kits were obtained from Cambridge Bioscience, UK and, unless otherwise stated, all reagents came from this kit. The Human PathwayFinder-1 GEArray kit was used (see Tables 4.1 and 4.2 for details). The first step was pre-hybridisation of the array membrane. Sheared herring sperm DNA (Promega, UK) was denatured at 100°C for 5 minutes and then quickly chilled on ice. This was then added to pre-warmed (68°C) GEHyb Hybridisation solution to a final concentration of 100μg DNA/ml and the mixture was maintained at 68°C in a water bath. The GEArray membrane was wetted with milli-Q water and placed in a hybridisation bottle. 10ml of the hybridisation solution was added to it and the membrane hybridised at 68°C for 1-2hrs with continuous agitation in a hybridisation oven. The remaining hybridisation solution was kept at 68°C for later use.

The next stage was to synthesise the probe. Total RNA was prepared using a commercially available kit (see section 2.6.2) and used as a template for [α-32P]cDNA probe synthesis. The first step was annealing. Total RNA (5μg), GEAcetamid mix (buffer A) (2μl) and RNase-free water (to a final volume of 20μl) were combined in a sterile PCR tube. The contents were mixed well by pipetting and centrifuged briefly. Tubes were then placed on a heat block at 70°C for 2min and cooled to 42°C, maintaining this temperature until the labelling mix was added. The labelling mix was prepared combining 5X concentrated GEAcetamid Buffer (Buffer B) (8μl), [α-32P]-dCTP (10mCi/ml) (5μl), RNase inhibitor (1μl) (Promega, UK), MMLV Reverse Transcriptase (50 units/μl) (2μl) (Perkin Elmer, UK) and RNase-free water (4μl). This mixture was then warmed to 42°C for 2mins and 20μl transferred to the annealing reaction. The contents were mixed thoroughly by pipetting and incubated at 42°C for 25mins. The labelling reaction was stopped by the addition of 10X concentrate Stop Solution (Buffer C) (5μl). The reverse transcribed cDNA probe was then denatured by adding 5μl of 10X concentrated Denaturing Solution (Buffer D) to the labelled cDNA probe and incubated at 68°C for 20mins. 50μl of 2X

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**Table 4.1** Human Pathway Finder-1 GEArray Kit. This table represents the location of the genes found on the array membrane. Each square on the grid is the equivalent to one spot on the membrane. See Table 4.2 for gene descriptions.
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<td>Stress</td>
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<tr>
<td>hsp27</td>
<td>Heat shock 27kD protein</td>
<td>Stress</td>
<td>4C, 4D</td>
</tr>
<tr>
<td>hsp90</td>
<td>Human mRNA for 90kDa heat shock protein</td>
<td>Stress</td>
<td>4E, 4F</td>
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<td>IκBα</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha</td>
<td>NFκB</td>
<td>5A, 5B</td>
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<td>IL-2</td>
<td>Interleukin 2</td>
<td>NFAT</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>5E, 5F</td>
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<td>mdm2</td>
<td>Mouse double minute 2, human homolog of p53-binding protein</td>
<td>p53</td>
<td>6A, 6B</td>
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<td>NFκB</td>
<td>6C, 6D</td>
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<td>Cyclin-dependent kinase inhibitor 2D</td>
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<td>Anti-proliferation/ TGFβ, p53</td>
<td>7A, 7B</td>
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<td>p53</td>
<td>Tumour protein p53</td>
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<td>7C, 7D</td>
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<td>Anti-proliferation/ TGFβ</td>
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<td>Bacterial plasmid</td>
<td>House keeping gene (negative control)</td>
<td>1G, 2G</td>
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Table 4.2 Gene descriptions for the Human Pathway Finder-1 GEArray Kit. The table also shows which signalling pathways are effected by these genes, as well as their grid position on the array membrane.
concentrated Neutralisation Solution (Buffer E) was then added and incubation continued for a further 10mins. The cDNA probe was now ready for the hybridisation solution. The pre-hybridisation solution was poured off the membrane and discarded. The denatured cDNA probe was mixed with the remaining hybridisation solution and added to the membrane. This was then hybridised overnight in the oven at 68°C.

The membrane was washed twice in wash solution 1 (2X concentrated SSC, 1%SDS) for 20mins at 68°C with agitation and twice in wash solution 2 (0.1X concentrated SSC, 0.5%SDS). The wet membrane was immediately wrapped in plastic wrap and exposed to X-ray film (Kodak MoMax MS-1) with an intensifying screen at −70°C for several days.

4.2 Results

4.2.1 Lactate Analysis of Human Explant Culture Supernatant

Osteoarthritic articular cartilage was obtained from a female, 74 year-old patient undergoing total knee replacement surgery. Cartilage explants were taken and cultured, as described. The culture supernatant was analysed using the lactate assay (Figure 4.1A). There was an observed increase in lactate release after the addition of IL-1β to the cultures but no other treatment had any effect and, hence, general metabolism was not significantly affected by the fatty acids.

4.2.2 DMBB Analysis of Human Explant Culture Supernatant

The same media described above was analysed using the DMBB assay to measure GAG release from the tissue to ensure the explant cultures were following characteristic patterns already observed with the addition of IL-1 and/or PUFAs. With the addition of IL-1β, there was an increase in GAG release into the media and, hence, proteoglycan degradation, which may have been reduced with n-3 PUFA supplementation, but not by n-6 PUFA (Figure 4.1B). Due to the nature of this, already, pathological tissue GAG release was significant even in untreated tissue.

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Figure 4.1 A) Lactate and B) GAG analysis of culture medium from human osteoarthritic cartilage obtained with ethical approval from a female, 74 year old patient undergoing a total knee replacement operation. Explant cultures were supplemented with or without n-3 (EPA) or n-6 (AA) polyunsaturated fatty acids (300µg/ml) overnight and in the absence or presence of IL-1β (10ng/ml) for 4 days. The lactate and DMMB assays were then carried out on the culture supernatant prior to RNA isolation and array investigations. (Mean ± S. D., n=2).
and no statistics could be carried out because only duplicate samples (n=2) were used from a single patient.

4.2.3 Human PathwayFinder-1 GEAarray Analysis

Total RNA was extracted from human explants, cultured as described in 4.1.1 and used to synthesise a radio-labelled cDNA probe prior to hybridisation to PathwayFinder-1 array membranes, as described in section 4.1.3. Each array is a positively charged nylon membrane spotted with 26 cDNA fragments from genes associated with several signal transduction pathways (mitogenic/ MAPK, stress, NFκB, NFAT, anti-proliferation, p53 and CREB signal pathway genes) that have been immobilised in duplicate (see Tables 4.1 and 4.2 for gene locations and descriptions).

Three RNA expression profiles were analysed and compared; control, IL-1β-treated and IL-1β plus n-3 (EPA)-supplementation (Figures 4.2-4.9). Overall, a general trend is seen for most genes, whereby, IL-1β treatment induces gene expression when compared to control sample RNA levels but, after addition of EPA, IL-1-induced-gene expression is abolished down to basal levels (Figure 4.2).

Using a two-fold change as relevant (i.e. a two-fold increase upon addition of IL-1β), we were able to make preliminary conclusions as to which pathways were affected. The c-fos and egr-1 immediate early genes (IEGs) are members of the mitogenic or MAPK pathway (Figure 4.3) and the expression levels of both were increased (at least two fold) in the presence of IL-1 and reduced back, almost to control levels on supplementation with EPA. However, these IEGs, c-fos and egr-1, are also members of the CREB pathway (Figure 4.9), hence, it is possible this pathway is also involved, although cyp 19 (another member of the CREB pathway) is not as dramatically affected by IL-1β.
Supplementation: For gene descriptions see Table 4.2.

Presence of IL-1β (10ng/ml) Con. control (no treatment); IL-1β supplemented samples; IL-1 + n-3, IL-1 β supplemented plus EPA

Cartilage explants were cultured with or without n-3 PUFAs (EPA, 300pg/ml) supplementation and in the absence or presence of human osteoarthritic articular cartilage (obtained as surgical waste from a female, 74 year old, total knee replacement genetral). From the Human Pathway Finder 1 (EF) array after hybridization with a cDNA probe

**Figure 4.2**: Expression patterns of all genes present on the Human Pathway Finder 1 (EF) array after hybridization with a cDNA probe
Figure 4.3 Expression levels of genes involved in the mitogenic signal pathway analysed using the Human PathwayFinder-1 GEArray (Cambridge Bioscience, UK).

Figure 4.4 Expression levels of genes involved in the stress signal pathway analysed using the Human PathwayFinder-1 GEArray (Cambridge Bioscience, UK).
c-myc, IκBα, iNOS and NFκB are all members of the NFκB signal pathway and each gene was
again, induced by approximately two-fold in the presence of IL-1β but reduced to near control
levels after treatment with n-3 PUFAs (EPA) (Figure 4.5). Another pathway possibly affected is
the TGFβ or anti-proliferation pathway (Figure 4.7). p19, p21 and p57 were all present on the
array, as representatives of this pathway, and once again, each gene was induced by IL-1β and
then induction was abolished upon EPA supplementation.

Although the other array genes follow a similar pattern of induction with IL-1β and consequent
reduction by EPA, it is difficult to make judgements as to whether they are affected genuinely.
The stress signal pathway marker genes (Figure 4.4) include ATF-2, c-fos, c-myc, hsf1, hsp 27,
hsp 90 and p53. Apart from heat shock protein 90 (hsp 90) all genes showed a general trend of
increased expression with IL-1β (although less than two-fold) with subsequent decreased
expression upon n-3 PUFA supplementation. Likewise, marker genes from the NFAT pathway
(CD5, fas ligand and IL-2) (Figure 4.6) followed the same trend. However, the increase in
expression was not more than two-fold compared to the control. The slightly IL-1-induced
expression of the fas ligand gene was reduced largely by supplementation with n-3 PUFAs to
much lower levels than the control. The p53 signal pathway was also represented on the array by
a wide selection of genes (p53, bax, gadd 45, mdm2, p21, pig 7 and pig 8) (Figure 4.8). For the
most part it seemed that no change (by two-fold) in gene expression was apparent (bax, gadd 45,
pig 7 and pig 8) with either IL-1β or n-3 PUFA. But, one result to notice is the expression of
mdm2 (p53 binding protein), although IL-1β did not seem to alter the mdm2 mRNA levels, upon
supplementation with EPA there was a large decrease in gene expression to below control levels.

The RNA analysed here was obtained from a single 74 year-old female patient and several
attempts were made to replicate these results. As previously mentioned in sections 3.2.2 and
3.2.4, human OA cartilage was already very pathological and difficult to maintain under culture
conditions. The tissue yields obtained from surgical waste donations were very poor and often the

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Figure 4.5 Expression levels of genes involved in the NFκB signal pathway analysed using the Human PathwayFinder-1 GEArray (Cambridge Bioscience, UK).

Figure 4.6 Expression levels of genes involved in the NFAT signal pathway analysed using the Human PathwayFinder-1 GEArray (Cambridge Bioscience, UK).
Figure 4.7 Expression levels of genes involved in the TGFβ/anti-proliferation signal pathway analysed using the Human PathwayFinder-1 GEArray (Cambridge Bioscience, UK).

Figure 4.8 Expression levels of genes involved in the p53 signal pathway analysed using the Human PathwayFinder-1 GEArray (Cambridge Bioscience, UK).
Figure 4.9 Expression levels of genes involved in the CREB signal pathway analysed using the Human PathwayFinder-1 GEAarray (Cambridge Bioscience, UK).
explant tissue or chondrocytes did not survive culture. By carrying out lactate and DMMB assays on culture supernatant it was possible to see that the condition of this cultured tissue was often poor. In addition, we were, unfortunately, unable to obtain normal, healthy human cartilage tissue due to a shortage of donors. Hence, only one patient was analysed successfully and the significance of the results cannot be assessed properly at present. Never-the-less, we have taken the observed increases and decreases (by at least two-fold) of certain gene expression levels as potential indicators of signalling pathways involved in cartilage metabolism, leading to further investigations of the MAPK pathways and NFκB pathways (see Chapters 5 and 6).

4.3 Discussion

The induction of immediate early genes (IEGs) following exposure to extracellular stimuli represents the first major transcriptional program that precedes changes in a variety of responses. The ERK, JNK and p38 kinase pathways are known to positively regulate IEGs, which results in various cellular outcomes, such as cell proliferation, differentiation and oncogenic transformation. Specifically the ERK1/2 signalling pathway plays a crucial role in IEG induction by directly activating IEG promoter-bound transcription factors and, hence, transcription of IEGs leading to the appearance of gene products such as Fos, Jun, Myc and Egr-1 (Murphy et al., 2004). Two such IEGs, c-fos and egr-1, were spotted on to the arrays studied in this chapter and used as marker genes for the mitogenic pathway (see Figure 4.3). The results indicated that mRNA from human articular cartilage explant cultures stimulated with IL-1β was highly expressed for both c-fos and egr-1 compared to control cultures, and, moreover, on addition of n-3 PUFA (EPA) this induced expression was reduced back to near control levels.

Previous studies have shown that egr-1 and c-fos can be induced by a wide variety of mitogens in a variety of cell types e.g. cytokines such as IL-1, IL-17 and TNFα (Granet and Miossec, 2004; Goetze et al., 2001; Chuen et al., 2004), growth factors such as fibroblast growth factor-2
(Santiago et al., 1999), UV radiation (Meyer et al., 2002) and lipopolysaccharide (LPS) (Pawlinski et al., 2003). Abnormal expression of either of these IEGs has been linked to various disorders including cancers (Wu et al., 2004; Baron et al., 2003), Alzheimer’s disease (Renbaum et al., 2003), rheumatoid arthritis (Tsuij et al., 2000) and osteoarthritis (Wang et al., 2000).

The cytokine, TNFα, is able to induce expression of c-fos and egr-1 in murine vascular smooth muscle cells and macrophages through extracellular signal-regulated kinases 1 and 2 (ERK-1 and -2) (Goetze et al., 2001). Studies showed that a specific inhibitor of the ERK1/2 pathways, PD98059, significantly inhibited the TNFα-induced expression of c-fos and egr-1 (Goetze et al., 2001). It is possible that IL-1β is having a similar effect in human chondrocytes as TNFα has in vascular smooth muscle cells and macrophages and, in fact, IL-1 has been shown to increase egr-1 in an osteoblast cell line (Granet and Miossec, 2004). Thus, it is possible that n-3 PUFAs are having an inhibitory effect on the ERK1/2 signalling pathway. It has also been shown that specifically inhibiting the ERK1/2 pathway can have anti-proliferative and anti-invasive effects on colon cancer in vitro and suppresses tumour growth in vivo (Sebolt-Leopold et al., 1999), so pharmaceutical or nutritional intervention of this pathway may also have beneficial effects in arthritis.

As previously described in section 1.15.1 the NFκB pathway has been implicated in a number of pathological situations including RA, cancer and Alzheimer’s disease. In the resting cell, NFκB is bound to and inhibited by IκBα which, when activated, dissociates from NFκB and allows translocation to the nucleus whereupon the transcription factor, NFκB, can activate gene transcription (Figure 1.17). A variety of cytokines are able to stimulate expression of NFκB (e.g. IL-1 and TNFα) and NFκB itself is able to increase the expression of cytokines and other genes involved in the inflammatory process such as iNOS and the inducible COX-2, both of which contribute to pathogenesis of inflammation. The c-myc oncogene (for review see Dang et al.,
1999) is reported to play a role in cell growth and differentiation. One previous study highlights the role of NFκB in mediating IL-1 induction of c-myc gene transcription in fibroblast cells (Kessler et al., 1992).

IκBα, NFκB, iNOS and c-myc were all present on the same arrays and the results also indicated an increase in expression after IL-1β treatment compared to untreated cultures. Moreover, this induction was reduced after supplementation with EPA (see Figure 4.5). This suggests that IL-1β is having an influence on inflammation propagation, a result that is in keeping with previous findings (reviewed by Bingham, 2002; Dinarello, 2000b). The effects of n-3 PUFA (EPA) on the NFκB pathway would suggest a possible target for the treatment of inflammatory diseases.

Glucosamine sulphate is a commonly used drug in the treatment of osteoarthritis and Largo et al., (2003) have recently shown that IL-1β-induced NFκB activation is reduced after glucosamine supplementation in human osteoarthritic chondrocyte cultures. It is possible then that EPA can affect the NFκB pathway in a similar manner and, hence, have beneficial anti-inflammatory properties.

In proliferating cells, the cell cycle consists of four phases starting at the Gap 1 (G1) phase, the interval between mitosis and DNA replication, characterised by cell growth. The transition that occurs at the restriction point (R) in G1 commits the cell to the proliferative cycle. If the conditions that signal this transition are not present, the cell exits the cell cycle and enters G0, a non-proliferative phase during which growth, differentiation and apoptosis occur. DNA replication occurs in the synthesis (S) phase, which is followed by G2 where growth and preparation for division occurs. Mitosis then occurs in the M phase.

Progression through the G1 phase and initiation of DNA synthesis (S phase) is regulated by several classes of cyclin dependent kinases (CDKs) (Figure 4.10), whose activities are in turn constrained by CDK inhibitors (CKIs). Two families of CKIs exist. The first are INK4 proteins...
Figure 4.10 Simplified diagram illustrating the anti-proliferation pathway or G1 to S phase checkpoint of the cell cycle. Red dashed arrows represent possible induction mechanisms. (Adapted from www.sigma-aldrich.com). Abbreviations: CDK, cyclin dependant kinase; Rb, retinoblastoma protein; E2F, transcription factor; P, phospho-group; cAMP, cyclic AMP.
(inhibitors of CDK4), which are able to inhibit catalytic subunits of CDK4 and 6. These include p16
$^{\text{INK4a}}$, p15$^{\text{INK4b}}$, p18$^{\text{INK4c}}$ and p19$^{\text{INK4d}}$. The second family includes p21$^{\text{Cip1}}$, p27$^{\text{Kip1}}$ and p57$^{\text{Kip2}}$, all of which are members of the CIP/KIP family whose actions effect the activities of cyclin D-, E- and A- dependent kinases (Sherr and Roberts, 1999). The CDK inhibitors p19$^{\text{INK4d}}$ (Chan et al., 1995; Hirai et al., 1995), p21$^{\text{Cip1}}$ (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993) and p57$^{\text{Kip2}}$ (Lee et al., 1995; Matsouka et al., 1995) were all present on the array and named p19, p21 and p57, respectively (see Figure 4.7).

Two cell cycle kinases, CDK4/6-cyclin D and CDK2-cyclin E, and the transcription complex that includes retinoblastoma protein (Rb) and the transcription factor, E2F, are pivotal in controlling the G1 to S phase checkpoint (Dyson, 1998). If DNA damage has occurred, p53 accumulates in the cell and induces p21-mediated inhibition of the CDK4/6-cyclin D complex. Similar inhibitory mechanisms include activation of TGFβ receptors that induce the inhibition of CDK4/6-cyclin D by p15, while cyclic-AMP inhibits CDK4/6-cyclin D via p27 (reviewed by Bartek and Lukas, 2001). If CDK4/6-cyclin D is inhibited, Rb is in a state of low phosphorylation and remains tightly bound to E2F, thus inhibiting its activity and preventing transition to the S-phase and DNA synthesis and further to the proliferation phase (Dyson, 1998).

The array data studied in this chapter showed that, in the presence of IL-1β, mRNA expression levels of p19, p21 and p57 increased, indicating that activation of the CDK-cyclin complex was inhibited and thus would prevent cell cycle progression from G1 to the S-phase but instead promote entry to the GO phase and possible apoptosis. However, when supplemented with EPA, expression levels of the above genes were reduced back to near basal levels, suggesting that the inhibitor concentration was reduced and, hence, the Rb-E2F complex would be phosphorylated allowing cell cycle progression to occur and, thus a reduction in apoptosis.

Schuerwegh et al., (2003) have shown that bovine chondrocyte proliferation can be inhibited by adding IL-1α, TNFα or IFNγ. However, pre-incubations with the anti-inflammatory cytokine, IL-
4 inhibits this reduction in proliferation as seen after treatment with the above pro-inflammatory cytokines. Recently, in vivo studies reported a decreased number of chondrocytes and an increased number of apoptotic chondrocytes in RA and OA cartilage (Kim and Song, 1999; Kouri et al., 2000; Goldring, 2000). Another recent study has shown that n-3 fatty acids (unspecified) significantly increase proliferation and reduce apoptosis in mucosal cells after small bowel graft transplantations in rats (Wu et al., 2003). These observations are consistent with my array results.

The mechanism by which n-3 fatty acids could promote proliferation and inhibit apoptosis are unclear but there are several suggestions; i) n-3 fatty acids can be acting as a substrate supplying an energy source for proliferation to the cells, ii) the n-3 fatty acids could be esterified into phospholipids and neutral lipids which are essential components of membrane structure for new cells, iii) it has been reported that IL-1β and TNF have possible pro-apoptotic effects (Steller, 1995), n-3 fatty acids could be inhibiting this and iv) it is possible that apoptosis could be a method of cell death carried out by mucosal cells after transplantation (Wu et al., 2003) or indeed by chondrocytes during culture and it is possible that n-3 fatty acids can inhibit apoptosis via immunosuppression (Kelley et al., 1989). The last two points would result in more specific effects and so it is more likely that n-3 PUFAs are using either or both of these modes of action.

Although the data discussed here is only obtained from one patient, it has enabled us to identify possible pathways (MAPK, NFκB and anti-proliferation) for further investigation (see Chapters 5 and 6).
4.4 Chapter Summary

- Neither IL-1β treatment nor PUFA (EPA or AA) supplementation had a significant effect on lactate release and, hence, cell viability and general metabolism in human OA articular cartilage explants obtained from a 74 year-old total knee replacement surgery patient.

- IL-1β induced GAG release from the same human cartilage explants was reduced by supplementation with a n-3 PUFA (EPA) but not with a n-6 PUFA (AA).

- Expression levels of marker genes from the MAPK pathway (c-fos and egr-1), the NFκB pathway (c-myc, IkBα, iNOS and NFκB) and the TGFβ/anti-proliferation pathway (p19, p21 and p53) were all up regulated in the presence of IL-1β by at least 2-fold.

- The IL-1-induced expression of the above genes was reduced back to basal levels after supplementation with the n-3 PUFA, EPA at 300μg/ml.

- If these results are reproduced with further patient samples, they may suggest that EPA acts via the above signalling pathways.
CHAPTER 5 - STUDIES USING SPECIFIC INHIBITORS OF INTRACELLULAR SIGNALLING PATHWAYS AND THEIR EFFECTS ON CARTILAGE METABOLISM

In view of the changed expression of signalling pathway genes shown in Chapter 4, further experiments were directed toward confirming the involvement of specific pathways with the n-3 PUFA effects. To do this we used specific inhibitors for key elements of specific pathways such as the MAPK cascades and the NFκB pathway.

Mitogen activated protein kinases (MAPKs) are proline-directed serine-threonine protein kinases that are activated by phosphorylation on both a threonine and tyrosine in a Thr-X-Tyr motif found in an activation loop proximal to ATP and substrate binding sites (section 1.15.2). Three main classes of MAPK are known, the ERKs, JNKs and the p38 kinases, all of which differ with respect to the size of the activation loop and the nature of the X in the Thr-X-Tyr motif, for example X is glutamic acid in ERKs, proline in JNKs and glycine in p38 kinases. Due to these variations in structure, each MAPK is activated by a different series of MAPK kinases (or MAPKKs) and has a different range of in vivo substrates.

The discovery of several ERK inhibitors has enabled investigation into this MAPK cascade. Two commercially available inhibitors of interest are PD98059 and U0126 (see Figure 5.1). PD98059 (2-[2-amino-3-methoxyphenyl]-4H-1-benzopyran-4-one) is a potent and selective cell-permeable inhibitor of MEK-1 (a MAPK kinase). It selectively blocks the activation of MEK, thereby inhibiting the phosphorylation and the activation of ERK. PD98059 has no effect on activated MEK-1 but does inhibit the activation of MEK-1 by Raf (MEK Kinase) (Alessi et al., 1995).

The second compound, U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene), is a potent specific inhibitor of both MEK-1 and MEK-2 in either in vitro studies or in vivo where U0126 blocks phosphorylation and activation of ERK-1 and ERK-2. Prevention of ERK activation would avert downstream phosphorylation of several factors including Elk-1 which in

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Figure 5.1 The chemical structures of SB203580, U0126, PD98059, Curcumin and PDTC, specific signal pathway inhibitors.

turn would stop induction of c-Fos and c-Jun, components of the AP-1 complex (Favata et al., 1998). U0126 is described in more detail in Chapter 6. Kinetic analysis of U0126 and PD98059 demonstrated that both compounds are non-competitive inhibitors with respect to both MEK substrates, ATP and ERK (Favata et al., 1998).

A series of pyridinyl imidazoles that inhibit the production of interleukin-1 and tumour necrosis factor from lipopolysaccharide-activated human monocytes have been found to bind to and inhibit p38 kinase. One example, SB203580 (4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]-1H-imidazole) (see Figure 5.1), is able to inhibit two splice forms of p38 as well as a homologue known as p38β (Jiang et al., 1996), but not the ERKs or JNKs or other serine-threonine protein kinases (Cuenda et al., 1995). Young et al. discovered that pyridinyl
imidazoles inhibit p38 kinase activity by binding to the ATP-binding site. Using *E.coli*-expressed p38 kinase, studies showed that SB203580 bound with a stoichiometry of 1:1 and that binding was blocked by pre-incubation of p38 kinase with an ATP analogue, FSBA (5'-[p-(fluorosulfonyl)benzoyl]adenosine) which covalently modifies the ATP-binding site. As expected from these results, the inhibition by SB203580 is competitive with ATP (Young et al., 1997). Thus, SB203580 is a useful tool for investigating the role of p38 kinase in regulating transcription, translation and cytoskeletal elements in response to various stress and cytokine stimuli as well as any potential role it may have in animal models of inflammatory disease (Saklatvala et al., 1996; Beyaert et al., 1996; Shapiro and Dinarello, 1995).

NFκB is a ubiquitous transcription factor that plays a major role in the cells of the immune system, where it controls the expression of various cytokines and major histocompatibility complex genes. NFκB proteins and other Rel family members reside in the cytoplasm when in an inactivated state and only translocate to the nucleus upon activation by various agents such as inflammatory cytokines (IL-1, TNF), mitogens, oxidative stress and UV light, among others (see section 1.15.1 for a more detailed description of the NFκB pathway).

Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione) (see Figure 5.1) is the major active component of tumeric (*Curcuma longa*) and is known for giving curry its specific flavour and yellow colour. Curcumin is also known to block many reactions in which NFκB plays a major role (Singh and Aggarwal, 1995). It is thought that curcumin blocks a signal upstream of NFκB-inducing kinase, IKK, and prevents IKK activation (Jobin et al., 1999).

Curcumin has been shown to display anti-carcinogenic properties in animal models. Studies have shown its ability to inhibit tumour initiation induced by carcinogens such as benz(α)pyrene and tumour promotion induced by phorbol esters (Khar et al., 1999; Huang et al., 1988; Huang et al., 1992). Curcumin has also been reported to inhibit type 1 human immunodeficiency virus long terminal repeat (HIV-LTR) directed gene expression and virus replication which requires NFκB.
activation (Li et al., 1993). Anti-inflammatory effects of curcumin have also been well
documented (Zhang et al., 1999; Abe et al., 1999). As well as NFκB inhibition, curcumin is also
known to inhibit activation of the JNK pathway and the transcription factor, AP-1. Inhibition of
JNK occurs by suppression of MEKK1-induced JNK activation (Chen and Tan, 1998).

Another NFκB inhibitor is ammonium pyrrolidinedithiocarbamate (PDTC) (Meyer et al., 1993)
(see Figure 5.1). PDTC is a thiol compound widely used to study the activation of redox
sensitive transcription factors e.g. NFκB and AP-1 (Wu and Momand, 1998). Studies have
shown that micromolar amounts of PDTC reversibly suppress the release of the inhibitory
subunit IκB from the latent cytoplasmic form of NFκB in cells treated with phorbol ester, IL-1 or
TNFα (Schreck et al., 1992).

The aim of the work described in this chapter was to supplement bovine chondrocyte monolayer
and explant cultures with each of these inhibitors (PD98059, U0126, SB203580, Curcumin and
PDTC) to enable preliminary investigations into signalling pathways associated with
chondrocyte metabolism and to identify possible pathways which could be investigated further to
elucidate some of the fatty acid-supplementation effects.
5.1 Materials and Methods

5.1.1 Signalling Pathway Inhibitor-Treated Explant Cultures

Bovine cartilage explants were obtained from bovine cartilage and pre-cultured as described in section 2.2.2. After the explants had been washed, inhibitors were added at various concentrations (see Table 5.1). After 30mins, the explants were supplemented with or without 10ng/ml IL-1α for 4 days at 37°C, 5% CO₂ and 95% air. The explants were then weighed, harvested and kept at −20°C prior to later use.

5.1.2 Lactate Assay

The lactate assay was used to evaluate the metabolic state of all culture media as previously described (see section 2.3). Assays were carried out to assure that the inhibitors (or their carrier solvents) were not causing a detrimental effect on tissue state. A significant decrease in lactate production during treatment was interpreted as a significant reduction in cell viability or metabolism.

5.1.3 DMBB Assay

DMBB assays were also carried out on all media collected. The protocol outlined in section 2.4 was used to analyse any effects the pathway inhibitors were having on GAG release from the tissue into the surrounding culture media.

5.1.4 Analysis of Endogenous Aggrecanase and MMP Activity using SDS-PAGE and Western Blotting Techniques

Endogenous aggrecanase or MMP activity on cartilage aggrecan in cell cultures was analysed using the explant culture supernatants as a source of cleaved proteoglycan. After previously carrying out the DMBB assay, media from each sample were analysed. The volumes of media
### Table A

<table>
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<tr>
<td>PD98059</td>
<td>2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one</td>
<td>C₁₆H₁₃NO₃</td>
<td>Prevents activation of MEK-1 by Raf hence inhibits the ERK-1 pathway</td>
</tr>
<tr>
<td>U0126</td>
<td>1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio)butadiene</td>
<td>C₁₈H₁₆N₆S₂</td>
<td>Inhibits both the active and inactive MEK-1 and MEK-2 to inhibit both ERK-1 and ERK-2 pathways</td>
</tr>
<tr>
<td>SB203580</td>
<td>4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole</td>
<td>C₂₁H₁₈N₄FOS</td>
<td>p38 Kinases</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione</td>
<td>C₂₁H₂₀O₆</td>
<td>NFκB, AP-1 and JNK (via MEKK1)</td>
</tr>
<tr>
<td>PDTC</td>
<td>Ammonium pyrrolidinedithiocarbamate</td>
<td>C₅H₉NS₂·NH₃</td>
<td>NFκB</td>
</tr>
</tbody>
</table>

### Table B

<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>Solvent used</th>
<th>Inhibitor/solvent stock concentration (mM)</th>
<th>Volume of inhibitor/solvent stock solution in culture media (µl/ml)</th>
<th>Final concentration of inhibitor used in cultures (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD98059</td>
<td>DMSO</td>
<td>30</td>
<td>0.25, 0.5, 1</td>
<td>7.5, 15, 30</td>
</tr>
<tr>
<td>U0126</td>
<td>DMSO</td>
<td>10</td>
<td>1.25, 2.5, 5</td>
<td>12.5, 25, 50</td>
</tr>
<tr>
<td>SB203580</td>
<td>DMSO</td>
<td>2.5</td>
<td>2, 4, 8</td>
<td>5, 10, 20</td>
</tr>
<tr>
<td>Curcumin</td>
<td>Ethanol</td>
<td>1</td>
<td>2.5, 5, 15</td>
<td>2, 5, 15</td>
</tr>
<tr>
<td>PDTC</td>
<td>Sterile water</td>
<td>100</td>
<td>0.5, 1, 2</td>
<td>50, 100, 200</td>
</tr>
</tbody>
</table>

**Table 5.1** Signalling pathway inhibitors. Table A) details the names and targets of each inhibitor whilst Table B) shows the concentrations used in culture. PD98059, U0126 and SB203580 were obtained from Alexis Biochemicals, UK. Curcumin and PDTC were obtained from Sigma-Aldrich, UK. Abbreviations: DMSO, dimethyl sulfoxide.
used were calculated to contain known amounts of GAG (30µg). Each media sample was deglycosylated with chondroitinase ABC (0.1U per 10µg of GAG) (Sigma-Aldrich, UK), keratanase (0.1U per 10µg of GAG) (MP Biomedicals, London, UK) and keratanase II (0.001U per 10µg of GAG) (also from MP Biomedicals, London, UK) at 37°C for at least 3 hours (Curtis et al., 2000). The samples were then dialysed in water overnight and lyopholised before they were reconstituted in equal volumes of 2X SDS sample buffer containing 10% v/v β-mercaptoethanol. Samples were run on 4-12% gradient SDS-PAGE gels and transferred electrophoretically onto nitrocellulose membranes as described in section 2.5.

BC-3 monoclonal antibody has been designed to recognise the amino-terminal neoeptipe sequence (ARGxx....) generated after aggrecanase catabolism within the-IGD of aggrecan (Hughes et al., 1995) (see Figure 5.2). Aggrecan catabolites, containing this neoeptipe, range in molecular mass from approximately 250kDa (which represents a singular aggrecan molecule), to around, 55kDa as aggrecanase cleaves at various sites along the core protein. The monoclonal antibody, BC-14, by contrast, is able to recognise the amino-terminal neoeptipe sequence (FFGxx....) generated after MMP catabolism of aggrecan within the IGD (Caterson et al., 1995) (see Figure 5.2). Western blotting was carried out using each primary monoclonal antibody (BC-3 and BC-14) at a concentration of 1:100 in 1% BSA overnight. The membranes were washed, an anti-mouse AP-conjugated secondary antibody (Promega, UK) applied (1:7,500 in 1% BSA), for 1 hour, washed again and developed using AP Buffer, BCIP and NBT (see section 2.5).

5.1.5 Statistical Analysis

The lactate and DMB assays were analysed using the Statview 4.02 software package for Mac. Bar graphs were generated showing the mean values and standard deviations and to test significance the ANOVA test was applied using a p-value cut off of 5%.

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Figure 5.2 Aggrecanase structure indicating aggrecanase and MMP cleavage points and recognition sites for monoclonal antibodies BC-3 (Hughes et al., 1995) and BC-14 (Caterson et al., 1995).

5.2 Results

5.2.1 Analysis of Cell Viability using the Lactate Assay

Bovine explant media was analysed after addition of either, PD98059, U0126, SB203580, Curcumin or PDTC. Each inhibitor was dissolved in a known solvent (DMSO, ethanol or water) prior to use in the culture systems. To be sure that the carrier solvent itself wasn't having a detrimental effect on the chondrocyte explant cultures, samples were set up with solvent alone (in the absence of inhibitor) and at the maximum levels used (see Table 5.1) and lactate assays carried out on the media collected from these (see Figure 5.3A). All other culture conditions remained exactly the same as with the experimental samples. It is clear that, although there was a slight increase of metabolism, and hence, lactate release in the IL-1-treated cultures, there was
Figure 5.3 Lactate and GAG analysis of cultures containing carrier solvents alone. Bovine articular cartilage explant cultures were treated with DMSO (8μl), ethanol (15μl) or water (2μl) in 1ml of DMEM, plus or minus IL-1α (10ng/ml) for 4 days. The volumes of carrier used are equivalent to the highest levels supplemented during inhibitor treatment. A) Lactate analysis and B) GAG analysis. (Means ± S.D.; n=4). No significant change was seen with carrier supplementation.

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no significant effect on lactate release with the addition of any of the three solvents compared to either the control or IL-1 alone samples.

Lactate assays were then carried out on all explant media (see Figure 5.4) collected from all experiments investigating different pathway inhibitors. IL-1-treated samples significantly increased metabolism compared to their comparative controls. With the introduction of any signalling pathway inhibitor there was generally no significant decrease in lactate release. With PD98059, U0126, SB203580, Curcumin or PDTC supplementation there was no significant decrease in lactate production in the IL-1-treated samples compared to IL-1 alone. In U0126 control samples (no IL-1α), the inhibitor seemed to cause a significant increase in lactate release. For a single concentration (10μM) of SB203580 there was a significant decrease compared to the control (no IL-1α).

5.2.2 Analysis of GAG Production from Cultures Supplemented with Inhibitors of Various Signal Pathways

First, DMMB assays were used to analyse media from explants with carrier solvent only treatment and no inhibitor supplementation (see Figure 5.3B). There was no statistically significant change in GAG release from the ECM into the surrounding medium with either DMSO, ethanol or water. The only significant change was a greatly increased GAG turnover seen once IL-1α was added to the culture, an observation that had been previously noted (Figure 5.2).

DMMB assays were carried out on all explant culture medium treated with pathway inhibitors. Bovine articular cartilage explant media was analysed after treatment with PD98059, U0126, SB203580, Curcumin and PDTC (Figures 5.5-5.9). The IL-1-induced increase in GAG release was not significantly changed in the presence of increasing doses of PD98059 (Figure 5.5). After U0126 supplementation there was a significant (p<0.0001) decrease in GAG release compared to
Figure S-4: Analysis of medium from bovine anterior ciliary explant cultures for lactate release. Samples were supplemented with or without Pd98059 (7.5-M), U0126 (12.5-M), SB203580 (5-20uM), or PDTC (50-200uM). Inhibitors were dissolved in their carriers and added to the explants 30 minutes prior to exposure with or without IFNg/ml II-1a for 4 days. Media was changed twice during the study. The data is presented as the mean ± S.D. of five determinations. Statistical analysis was performed using one-way ANOVA followed by post-hoc analysis (Fisher’s least significant difference). The p-value was calculated using OriginPro 8 software. Significant differences are indicated with an asterisk (*) and a plus sign (+).
Figure 5.5 GAG analysis of PD98059-supplemented bovine explant media. Samples were supplemented with or without PD98059 (7.5-30µM) dissolved in DMSO, prior to exposure with or without 10ng/ml IL-1α for 4 days. Media was analysed using the DMMB assay (see section 2.4). (Means ± S. D.; n=9).
the IL-1-induced samples or IL-1 plus DMSO only samples. However, this decrease after U0126 did not appear to be dose-dependent and similar amounts of inhibition occurred at all doses of U0126 (12.5-50μM) (Figure 5.6). There was a significant (p<0.015) increase of GAG release into the media with high concentrations of SB203580 (10-20μM) compared to IL-1 alone or IL-1 plus DMSO treated explants (Figure 5.7). Upon treatment with Curcumin there appeared to be a decrease in IL-1-induced GAG release with 2μM Curcumin although with higher doses (5-15μM) this effect was not seen, when compared to IL-1 alone samples. However, there also appeared to be a slight but significant (p<0.04) decrease in GAG release upon addition of the carrier, ethanol (Figure 5.8), although the addition of ethanol at the concentrations used here had been shown previously to have no significant effect on these cultures (Figure 5.3). Lastly, the addition of PDTC to this culture system appeared to have a similar effect as U0126 with a highly significant (p<0.0001) decrease in GAG release at all concentrations (50-200μM) compared to IL-1 alone cultures which was not dose-dependent (Figure 5.9).

5.2.3 Analysis of Endogenous Aggrecanase Activity on Aggrecan Degradation using Western Blotting with the BC-3 Monoclonal Antibody

Bovine cartilage explants were cultured as per section 5.1.1 and culture supernatant collected. SDS-PAGE and Western blotting techniques were then used to analyse the endogenous aggrecanase activity on cartilage aggrecan. The monoclonal antibody BC-3 was used to specifically recognise the cleaved amino terminal epitope sequence (ARGxx) (Hughes et al., 1995) (see Section 5.1.4).

In all cases, bovine explant media showed a large increase in aggrecanase metabolites after stimulation with IL-1α. Aggrecanase positive bands can be seen creating a smear between 250kD (representing the whole aggrecan core protein) and 55kD indicating the presence of various molecular weight products of aggrecan catabolism by aggrecanase. However treatment
Figure 5.6 GAG analysis of U0126-supplemented bovine explant media. Samples were supplemented with or without U0126 (12.5-50μM) dissolved in DMSO, prior to exposure with or without 10ng/ml IL-1α for 4 days. Media was analysed using the DMMB assay (see section 2.4). (Means ± S. D.; n=12). *=p<0.0001 versus IL-1 alone.
Figure 5.7 GAG analysis of SB203580-supplemented bovine explant media. Samples were supplemented with or without SB203580 (5-20µM) dissolved in DMSO, prior to exposure with or without 10ng/ml IL-1α for 4 days. Media was analysed using the DMMB assay (see section 2.4). (Means ± S. D.; n=9). *=p<0.015 versus IL-1 alone.
Figure 5.8 GAG analysis of Curcumin-supplemented bovine explant media. Samples were supplemented with or without Curcumin (2-15µM) dissolved in ethanol, prior to exposure with or without 10ng/ml IL-1α for 4 days. Media was analysed using the DMMB assay (see section 2.4). (Means ± S. D.; n=9). *=p<0.005 and ‡=p<0.04 versus IL-1 alone.
Figure 5.9 GAG analysis of PDTC-supplemented bovine explant media. Samples were supplemented with or without PDTC (50-200μM) dissolved in water, prior to exposure with or without 10ng/ml IL-1α for 4 days. Media was analysed using the DMMB assay (see section 2.4). (Means ± S. D.; n=12). *p<0.0001 versus IL-1 alone.
with the specific signal pathway inhibitors had varying results on aggrecanase activity. In the presence of increasing doses of PD98059, the IL-1-induced aggrecanase activity appeared to be unchanged (Figures 5.10A and B). There appeared to be an increase in aggrecanase activity in media treated with IL-1 plus DMSO (Figure 5.10B, lane 7), although this increase was not seen in the other inhibitor-treated samples (PD98059 was dissolved in DMSO) (Figure 5.10B, lanes 8-10). Analysing the U0126 media showed that the IL-1-induced aggrecanase activity (Figure 5.11A, lane 4; Figure 5.11B, lanes 6 and 7) was greatly reduced in the presence of 25-50μM U0126 (Figure 5.11A, lanes 5 and 6; 5.11B, lanes 9 and 10). Upon treatment with SB203580, IL-1-induced aggrecanase activity seemed to increase further with all concentrations of SB203580 (5-20μM) (Figure 5.12A, lanes 5 and 6; Figure 5.12B, lanes 8-10) compared to IL-1 alone (Figure 5.12A, lane 4; Figure 5.12B, lane 6) and IL-1 plus DMSO (Figure 5.12B, lane 7), this is in agreement with the increase in GAG release observed (Figure 5.7). Figure 5.13 shows the effects of curcumin inhibition on aggrecanase activity. Here, curcumin may have reduced aggrecanase activity at the highest dose of 15μM (Figure 5.13A, lane 6), although this decrease was not seen in the repeated experiment (Figure 5.13B). Finally, 200μM PDTC appeared to reduce IL-1-induced aggrecanase activity in bovine articular cartilage explants (Figure 5.14A, lane 6; Figure 5.14B, lane 10). All experiments have been repeated at least three times for all inhibitors, obtaining similar results (two representative experiments are shown in each of figures 5.10-5.14).

5.2.4 Analysis of Endogenous MMP Activity using the BC-14 Monoclonal Antibody

Media from bovine articular cartilage explants was also analysed using SDS-PAGE and Western blotting with monoclonal antibody BC-14 which specifically recognises MMP cleavage of aggrecan within the IGD of the core protein. The antibody recognises an amino-terminal neoepitope with the sequence FFGxx (Caterson et al., 1995). However, in all the supernatants

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Figure 5.10 Western blot analysis of PD98059-treated bovine explant media. Samples were supplemented with or without PD98059 [A] 15-30μM B) 7.5-30μM plus carrier (C) plus or minus 10ng/ml IL-1α for 4 days. Media was analysed using MAb BC-3 specific for aggrecanase cleaved amino terminal neoepitope sequence (ARGxx).
Figure 5.11 Western blot analysis of U0126-treated bovine explant media. Samples were supplemented with or without U0126 [A] 25-50μM B) 12.5-50μM plus carrier (C)] plus or minus 10ng/ml IL-1α for 4 days. Media was analysed using MAb BC-3 specific for aggreganase cleaved amino terminal neoepitope sequence (ARGxx).
Figure 5.12 Western blot analysis of SB203580-treated bovine explant media. Samples were supplemented with or without SB203580 [A] 10-20μM B) 5-20μM plus carrier (C)] plus or minus 10ng/ml IL-1α for 4 days. Media was analysed using MAb BC-3 specific for aggrecanase cleaved amino terminal neoepitope sequence (ARGxx).
**Figure 5.13** Western blot analysis of curcumin-treated bovine explant media. Samples were supplemented with or without curcumin [A] 5-15μM B) 2-15μM plus carrier (C)] plus or minus 10ng/ml IL-1α for 4 days. Media was analysed using MAb BC-3 specific for aggrecanase cleaved amino terminal neoepitope sequence (ARGxx).
Figure 5.14 Western blot analysis of PDTC-treated bovine explant media. Samples were supplemented with or without PDTC (A) 100-200μM B) 50-200μM plus carrier (C)) plus or minus 10ng/ml IL-1α for 4 days. Media was analysed using MAb BC-3 specific for aggrecanase cleaved amino terminal neoepitope sequence (ARGxx).

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analysed there was no positive banding present indicating that there was no MMP cleavage of aggrecan in these bovine cartilage explant cultures (results not shown).

5.3 Discussion
The preliminary research described in this chapter using commercially available inhibitors which were selective for specific signalling pathways, identified possible targets that parallel the actions of n-3 PUFAs (see Chapter 4). PD98059 and U0126 are both inhibitors of the ERK pathway. However, the results obtained varied with the two compounds. Both PD98059 and U0126 were used to investigate the effects of ERK inhibition on cartilage aggrecanase activity and glycosaminoglycan (GAG) release from the tissue into the culture media. With PD98059 supplementation there was no significant effect on IL-1α-induced GAG release or aggrecanase activity measured using the DMMB assay and Western blotting with monoclonal antibody BC-3, respectively. However, after addition of U0126 to the same cultures there was a marked decrease in both these markers of aggrecan catabolism.

Each inhibitor (PD98059 or U0126) acts on the same pathway but has different modes of action. PD98059 is able to inhibit the activation of MEK-1, upstream of ERK-1, whereas U0126 is able to bind to and specifically inhibit both inactive and active forms of MEK-1 and MEK-2 (Goueli et al., 1998) (described in more detail in Chapter 6). It is possible then that MEK-2 and the ERK-2 signalling pathway plays a role in the processes involved in IL-1-induced aggrecan catabolism and, hence, this is not inhibited by PD98059.

Further work investigating the effects of ERK pathway inhibition by U0126 may increase the understanding of this pathway and its role in cartilage metabolism (see Chapter 6).

The p38 MAPKs are a family of serine/threonine protein kinases that play important roles in cellular responses to external stress signal e.g. UV light, heat, osmotic shock and inflammatory
cytokines. Several p38 MAPK inhibitors have been shown to block IL-1 and TNF production (Adams et al., 2001). Inhibition of cytokine production seems to result from combined effects at the levels of both transcription and translation. In addition, p38 is involved in the induction of several other inflammatory molecules e.g. COX-2 (Guan et al., 1998) and iNOS (Badger et al., 2000). This points to a role of p38 kinases in inflammatory diseases and, indeed, studies have shown p38 MAPK inhibitors to be efficacious in animal models of rheumatoid arthritis i.e. collagen-induced arthritis in the mouse (Badger et al., 1996).

A p38 MAPK inhibitor, SB203580, an imidazole compound, was originally designed as a pharmacological tool for investigating cytokine regulation (Gallagher et al., 1995). In our work SB203580 was used to investigate the effects of p38 MAPK inhibition on the catabolism of bovine articular cartilage. We found that both GAG release from the tissue and aggrecanase activity was increased in the presence of SB203580. Other preliminary work using SB203580 suggests possible decreases in IL-1α-induced COX-2 protein levels, as well as lowered expression of IL-1α-induced COX-2, MMP-3, MMP-13, aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) mRNA isolated from bovine cartilage explant tissue, although this work needs to be repeated to confirm whether this is a true effect of p38 MAPK inhibition (data not included). It is unclear why aggrecanase activity should be enhanced if aggrecanase gene expression is decreased. Further work is needed to confirm and then explain this. Another previous study showed that SB203580 repressed TGFβ-induced aggrecan gene expression in a dose-dependent manner in a chondrogenic cell line (Watanabe et al., 2001).

As p38 MAPK inhibitors are able to block the production of inflammatory cytokines it is likely that the use of such compounds in diseases where such cytokines are elevated may be beneficial e.g. Alzheimer's disease, ischaemia, arthritis and inflammatory bowel disease. Imidazole analogues based on the structure of SB203580 are reportedly being tested in human clinical trial (reviewed by Kumar et al., 2003).
NFκB is a transcription factor known to play a role in the induction of genes involved in the response to injury and inflammation, among others. The specific inhibitor PDTC, a dithiocarbamate, is an antioxidant and a potent inhibitor of NFκB. Therefore, treatment with PDTC should reduce inflammation. Cuzzocrea et al. (2002) tested this theory in animal (mouse) models of collagen-induced arthritis and have published several findings. Results of interest include a reduction in iNOS activity and nitric oxide production, a reduction in IL-1β and TNFα levels, a lowered expression of COX-2 and also the prevention of IκBα degradation and, hence, translocation of NFκB from the cytoplasm and into the nucleus. They concluded, therefore, that prevention of activation of NFκB by PDTC reduces the development of acute and chronic inflammation. The results obtained in this chapter show that PDTC was able to reduce both GAG degradation and aggrecanase activity in cleaving the large proteoglycan, aggrecan of bovine articular cartilage. This implies that the NFκB transcription factor and signalling pathway may play a role in the processes involved in propagating cartilage degradation and, hence, inhibition of NFκB may represent an approach towards the treatment of inflammation and inflammatory diseases. It is also possible that n-3 PUFAs are having an effect on the NFκB pathway. Previous results have shown that these fatty acids have an effect on reducing COX-2 message, protein and activity (Chapter 3) iNOS and NFκB gene expression (Chapter 4) and also IL-1β and TNFα mRNA levels (Curtis et al., 2000; 2002).

Curcumin is an inhibitor of the NFκB pathway, as well as the JNK pathway and AP-1 transcription factor. In our studies no significant change in GAG release or aggrecanase activity was observed in the bovine cartilage explant cultures used. It is possible that the concentrations of curcumin supplemented in the culture medium were not sufficient enough to have an effect on the NFκB transcription factor. One study, used a 5-15μM concentration range of curcumin in the pre-treatment of chondrocytes prior to IL-1 stimulation (Liacini et al., 2002). They recorded a significant suppression of MMP-3 and MMP-13 mRNA expression in both human and bovine...
chondrocytes, which was attributed to the inhibition of the JNK pathway and its downstream target AP-1 since both the MMP-3 and MMP-13 promoters contain AP-1 transcription factor binding sites. Thus, it is possible that the concentration range used in our work (2-15μM) is not sufficient to inhibit the NFκB pathway and, hence, reduce GAG release and aggrecanase activity in the same manner as PDTC. Further work using larger concentration ranges of curcumin would need to be carried out to supplement these preliminary studies and see if this is indeed the case.

On the basis of these results, further work was begun to investigate the effects of several of these pathway inhibitors (U0126, SB203580 and PDTC) on other aspects of cartilage metabolism such as the degradative enzymes (MMP-3, MMP-13, aggrecanase-1 [ADAMTS-4] and aggrecanase-2 [ADAMTS-5]) and inflammatory and lipid mediators such as COX-2 and PGE_2. The results of preliminary work involving SB203580 have been previously mentioned above while studies using PDTC have yielded inconclusive results (data not shown). However, experiments using the ERK pathway inhibitor U0126 are promising (see Chapter 6).

5.4 Chapter Summary

- None of the carrier solvents (DMSO, ethanol or water) used for inhibitor studies had a significant effect on lactate release or GAG release in articular cartilage chondrocytes at the concentrations used in these studies.

- There were no significant changes of lactate release indicating that exposure to PD98059 (ERK-1), U0126 (ERK-1 and ERK-2), SB203580 (p38 kinases), Curcumin (NFκB, AP-1 and JNK) and PDTC (NFκB) were detrimental to cartilage metabolism.

- The ERK-1 signal pathway inhibitor, PD98059 appeared to have no effect on GAG release or aggrecanase cleavage of aggrecan.
• The ERK-1 and -2 signal pathway inhibitor, U0126 did decrease both the IL-1-induced GAG release and the activity of aggrecanase in manner suggesting similarities with n-3 PUFA effects (see Chapter 6).

• Inhibition of the p38 kinases using SB203580 seemed to increase both GAG release and aggrecanase activity.

• The NFkB, AP-1 and JNK pathway inhibitor, curcumin had no effect on GAG release or aggrecanase cleavage of aggrecan.

• The addition of PDTC, a NFkB specific inhibitor did result in a decrease in both IL-1-induced GAG release and aggrecanase activity.
CHAPTER 6 - STUDIES USING THE ERK PATHWAY SPECIFIC INHIBITOR, U0126 AND COMPARISONS WITH n-3 POLYUNSATURATED FATTY ACID EFFECTS ON CARTILAGE METABOLISM

U0126 is a potent MEK (or ERK kinase) inhibitor, which inhibits activation of MAPK by ERK both in vitro and in vivo (Favata et al., 1998; DeSilva et al., 1998). U0126 is an organic compound (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) that has been identified as an inhibitor of AP-1 transactivation in cell-based reporter assays (Favata et al., 1998). This compound is able to inhibit endogenous promoters containing AP-1 response elements but has no effect on genes that lack AP-1 response elements. These effects of U0126 are a result of direct inhibition of MEK-1 and MEK-2 (see Figure 6.1).

Although both U0126 and PD98059 appear to exhibit similar mechanisms of MEK inhibition, the mode of action of U0126 is distinct from that of PD98059. PD98059 binds only to the inactive (non phosphorylated) form of MEK-1, its binding being mediated by the conformational changes that accompany activation of the enzyme, which lead to inhibition of MEK-1 activation by Raf (Dudley et al., 1995; Alessi et al., 1995). Unlike PD98059, U0126 inhibits MEK-2 as well as MEK-1 and is capable of directly inhibiting activated MEK-1 and preventing endogenously active MEK-1 and -2 from phosphorylating and activating ERK-1 and -2. Therefore, U0126 also acts one step further downstream of PD98059 and blocks activity of MEK as well as its activation (Goueli et al., 1998) (see Figure 6.1). U0126 inhibition is selective for MEK-1 and -2 and it has been proven to have no effect on the kinase activity of protein kinase C (PKC), Raf, MEKK, ERK, JNK or several other related kinases (Favata et al., 1998). The MEK affinity of U0126, its selectivity for MEK over other kinases and its effectiveness in cellular environments suggests that this compound can serve as a powerful tool for in vitro and cellular investigations of MAPK-mediated signal transduction (Favata et al., 1998).

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Figure 6.1 Schematic showing the sites of inhibition of U0126 and PD98059 within the extracellular signal-regulated kinase (ERK) signalling pathway. PD98059 acts by inhibiting the activation of MEK-1 and -2 while U0126 also acts directly upon MEK-1 and -2 (adapted from Goueli et al., 1998). Elk-1, a ternary complex factor transcription factor containing an Ets domain; ATF-1, activating transcription factor-1.
The aim of the studies described in this chapter was to use U0126 to investigate the expression of degradative enzymes and inflammatory factors associated with arthritis and to compare these results with those found in previous studies using $n$-3 polyunsaturated fatty acids (Chapters 3, 4 and Curtis et al., 2000; 2002).

6.1 Materials and Methods

6.1.1 U0126-Treated Bovine Chondrocyte Cultures

Chondrocytes were obtained from bovine cartilage as described in section 2.1.1 and were either plated at $2 \times 10^6$ cells/well in 6 well plates or at $6 \times 10^6$ cells/well in 60mm Petri dishes. The cells were left to settle for a few hours at 37$^\circ$C, 5% CO$_2$ and 95% air. An ERK specific signal pathway inhibitor, U0126 was added at various concentrations (see Table 5.1) and left for 30mins before supplementation with or without 10ng/ml IL-1$\alpha$ for 24 hours. The cells were then harvested and kept at -20$^\circ$C until later use.

Explant cultures were also established using articular cartilage from 7-day old bovine metacarpal and metatarsophalangeal joints and pre-cultured as described in section 2.2.2. The explants were washed and U0126 added, left for 30min and supplemented with or without 10ng/ml IL-1$\alpha$ for 4 days. The explants were then weighed, harvested and kept at -20$^\circ$C until later use.

6.1.2 SDS-PAGE and Western Blot Analysis of Phosphorylated Extracellular Signal-Regulated Kinase-1 and -2 (ERK-1 and -2)

Phospho-p44/42 MAP Kinase (Thr202/Tyr204) antibody is a commercially available polyclonal antibody specific for the dually phosphorylated active forms of the ERK-1 and ERK-2 enzymes (Cell Signalling Technology, New England Biolabs, USA).

Monolayer cultures were grown in 60mm culture plates and treated as described previously (section 6.1.1). After the culture period, cells were lysed in 2X concentration sample buffer
(0.125M Tris HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue) and scraped up using a rubber policeman and transferred into an Eppendorf tube (for storage at −20°C, until later use). Equal volumes of the whole cell preparation were loaded into wells of 10% SDS gels and electrophoresis carried out as described in section 2.5. Western blotting was carried out using the primary antibody specific for phospho-p44/42 MAP Kinase (Thr202/Tyr204) at a concentration of 1:1000 in 1% BSA, overnight, followed by an AP-conjugated anti-rabbit secondary antibody (Sigma Aldrich, UK) at a concentration of 1:3000 in 1% BSA for 1 hour. The membranes were washed, substrate added and developed.

6.1.3 Analysis of Endogenous Aggrecanase Activity using DMMB Assay, SDS-PAGE and Western Blotting Techniques

The DMMB assay was used to investigate the levels of GAG released from bovine cartilage explant cultures into the surrounding media after supplementation with U0126 in the absence or presence of IL-1α following the protocol described in section 2.4.

Endogenous aggrecanase activity in cell cultures was also analysed using the explant culture supernatants as a source of cleaved proteoglycan. After previously carrying out the DMMB assay, media from each sample was analysed for endogenous aggrecanase activity as detailed in section 5.1.4. The supernatant was subjected to chondroitinase ABC, keratanase and keratanase II digestion, prior to SDS-PAGE and Western blotting using the monoclonal antibody BC-3 specific for the aggrecan generated amino-terminal neoepitope sequence (ARGxx....) from within the IGD of aggrecan (Hughes et al., 1995).
6.1.4 RT-PCR using Oligonucleotides Specific for Various Degradative Enzymes and Inflammatory Factors

Total RNA was extracted from bovine articular cartilage explants as described in section 2.6 and oligonucleotide primers were designed to specifically recognise bovine COX-2, MMP-3, MMP-13, TIMP-1, TIMP-2, TIMP-3, Aggrecanase-1 (ADAMTS-4), Aggrecanase-2 (ADAMTS-5), Aggrecan, and GAPDH was used as a house keeping gene for comparison. RT-PCR and sequence analysis was carried out as described in sections 2.8 and 2.9. Primer sequences and RT-PCR conditions are outlined in Table 2.1.

6.1.5 Cyclooxygenase Protein Extraction

As previously outlined in section 3.1.5 protein extraction was carried out prior to Western blot analysis of COX-2 protein.

6.1.6 Western Blot Analysis of Cyclooxygenase-2 Protein Levels in U0126-Treated Monolayer Cultures

Reduced samples were electrophoresed on 10% polyacrylamide gels in SDS following the standard protocol outlined in section 2.5, transferred to nitrocellulose membranes and blocked with 5% BSA/TSA. The membranes were immunoblotted with a polyclonal primary antibody, goat anti-COX-2 (Santa Cruz Biotechnology, USA), overnight. Both primary antibodies were used at 1:1000 diluted with 1% BSA. The primary antibody was removed, the membranes washed with TSA and an alkaline-phosphatase conjugated anti-goat secondary antibody (Sigma Aldrich, UK), was added to the membrane at a concentration of 1:10,000 in 1% BSA, for 1 hour. The membrane was washed, substrate added and developed as previously described (see section 2.5).
6.1.7 PGE₂ ELISA Immunoassay

The PGE₂ immunoassay kit was used to calculate PGE₂ concentrations present in bovine explant media after U0126 inhibitor treatment as described in section 3.1.7. Statistical significance was evaluated using Statview 4.02 software package for Mac to carry out an ANOVA test, and Microsoft Excel (Office 2001).

6.2 Results

6.2.1 Western Blot Analysis to Confirm Inhibition of the ERK Signalling Pathway by U0126

Using a polyclonal antibody specific to the active forms of ERK-1 and -2 (44kD and 42kD respectively) it is clear that addition of the inhibitor U0126 (12.5-50μM) to bovine articular cartilage chondrocyte monolayers resulted in a down-regulation in phosphorylated ERK-1 and -2 proteins (see Figure 6.2, lanes 3-5, 9 and 10). It is unclear as to whether IL-1α had an effect on inducing ERK activation but it seemed that a higher concentration of U0126 (25μM at least) was required to have an effect on preventing ERK activation in IL-1 treated cultures (lane 9), whereas in control cultures 12.5μM of U0126 was sufficient to inhibit this activation (lane 3).

6.2.2 Analysis of Glycosaminoglycan Release and Endogenous Aggrecanase Expression and Activity in U0126-Treated Bovine Articular Cartilage Explant Cultures

Bovine cartilage explant cultures were supplemented with U0126 (25-50μM) in the absence or presence of IL-1α. Conditioned media was analysed by DMMB assay for GAG release and by Western blot analysis using monoclonal antibody BC-3 to specifically recognise aggrecanase cleavage of aggrecan (see Figure 6.3).

Untreated bovine articular explant cultures (or controls, Figure 6.3A, lanes 1-3) released very little GAG from the tissue into the supernatant, whilst IL-1 induced a massive release of GAG (lane 4). When supplemented with 25μM U0126 (lane 5) there was a significant reduction in the
Figure 6.2 Western blot analysis using a polyclonal antibody specific to the dually phosphorylated and active 44kDa and 42kDa ERK-1 and -2 isoforms. Bovine chondrocyte monolayers were cultured with or without 12.5-50μM U0126 (added in DMSO), in the absence or presence of IL-1α for 24 hours.
Figure 6.3 Analysis carried out on conditioned bovine explant media supplemented with or without U0126 (25-50μM) in the absence or presence of IL-1α (10ng/ml) for 4 days. A) GAG release measured using the DMMB assay (Mean ± S. D.; n=3, *p<0.01) B) Western blot analysis using monoclonal antibody BC-3 which recognises aggrecanase cleavage of aggrecan.

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concentration of GAG present in the media. However, when treated with a higher dose of U0126 (50μM) there was no further decrease in GAG release (lane 6), indicating that only about half of the increase in GAG release was susceptible to the inhibitor’s action. This result was supported by a similar decrease in BC-3 positive aggrecanase activity (Figure 6.3B). The IL-1 induced presence of aggrecanase metabolites (lane 4) was decreased in the presence of 25μM U0126 (lane 5). As above, the higher dose of U0126, 50μM, did not seem to have an effect over and above that of the lower dose. It appears then that there is a limit to the effect that U0126 has on the IL-1-induced cartilage breakdown in the bovine articular explant culture system, as measured by either GAG release or aggrecanase activity. Next, total RNA was isolated from bovine cartilage explants and RT-PCR carried out to investigate the mRNA expression patterns of various degradative enzymes and inflammatory factors associated with OA. Oligonucleotide primer pairs (Table 2.1) were designed to investigate the expression of aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) (see Figure 6.4). The presence of IL-1 in the culture medium led to a strong increase in mRNA levels for both forms of aggrecanase. With the supplementation of U0126 (25-50μM) there was a marked reduction in aggrecanase-1 mRNA expression (lanes 4-6). This reduction appeared to be dose-dependent and with 50μM U0126 was reduced to basal levels. In contrast, no pattern of reduction was seen with aggrecanase-2 mRNA expression after U0126 treatment (lanes 4-6). It is interesting to note that GAG release and endogenous aggrecanase activity were not abrogated completely upon addition of U0126, hence, it is possible that U0126 is only targeting aggrecanase-1 and the aggrecanase-2 enzyme may still be active.

Aggrecan mRNA levels were also investigated. Aggrecan appeared to remain constitutive with no effects of IL-1 or U0126 treatment indicating that aggrecan synthesis was unaffected by this inhibitor. Further analysis should be carried out using real-time PCR to deduce whether this is a quantitative result, as reverse transcription (RT)-PCR is only a qualitative experiment. However,
**Figure 6.4** RT-PCR analysis of mRNA expression patterns of degradative and inflammatory factors after U0126 treatment. Total RNA was isolated from 7-day old bovine articular cartilage explants supplemented with or without U0126 (25-50μM) and in the presence or absence of IL-1α (10ng/ml) for 4 days. One representative experiment is shown here.
all amounts of RNA used within the experiment were normalised to the constitutive housekeeping gene, GAPDH.

6.2.3 Analysis of Cyclooxygenase-2 mRNA Expression, Protein Production and Activity in U0126-Treated Bovine Chondrocyte Cultures

RT-PCR, Western blotting and ELISA immunoassay techniques were used to investigate the effects of U0126 on the expression, protein levels and activity of cyclooxygenase-2. RT-PCR was carried out using oligonucleotide primers specific to bovine cyclooxygenase-2 mRNA (see Table 2.1). The previously reported IL-1 induction of cyclooxygenase-2 (section 3.2.3) was down regulated in the presence of U0126 in a dose-responsive manner, with the 50μM dose having the greatest affect, reducing COX-2 expression to control levels (Figure 6.4, lanes 4-6). Western blot analysis using a polyclonal antibody to COX-2 (Figure 6.5) also showed the same pattern. IL-1-induced COX-2 protein levels were abrogated in an apparently dose-dependent manner upon U0126 (25-50μM) addition to the bovine chondrocyte monolayer cultures (lanes 4-6). This experiment has been carried out on three separate occasions and similar results were observed each time.

PGE₂ production was also investigated using ELISA techniques previously described in section 3.1.7. IL-1α increased the levels of PGE₂ present in the explant media compared to the control media by approximately 2000 fold, but supplementation with U0126 significantly reduced this (Figure 6.6). This experiment was repeated three times with triplicate assays for each sample and similar results were obtained for each experiment, as indicated by the standard deviations displayed in Figure 6.6. These results indicate that IL-1 induced COX-2 message, protein and, hence, release of the COX-2 product, PGE₂ were all inhibited strongly in the presence of the ERK pathway inhibitor U0126 in bovine chondrocyte cultures.
Figure 6.5 Western blot analysis of COX-2 protein from bovine monolayer chondrocytes supplemented with or without U0126 (25-50μM) and in the absence or presence of IL-1α (10ng/ml) for 24 hours.
Figure 6.6 PGE$_2$ release measured using an ELISA Immunoassay kit (R & D Systems, UK). Media surrounding bovine articular cartilage explants supplemented with or without U0126 (25-50µM) and in the absence or presence of IL-1α (10ng/ml) for 24 hours. (Mean ± S. D., n=3) Three separate experiments with triplicate readings taken from each sample. *p<0.0001 compared to IL-1 treatment alone.
6.2.4 Analysis of MMP-3 and -13, TIMP-1, -2, and -3 mRNA Expression in U0126-Treated Bovine Explant Cultures

RT-PCR was used to analyse the expression levels of MMP-3 and -13 as well as their inhibitors, TIMPS-1, -2 and -3. For details of primer sequences and PCR conditions see Table 2.1. Total RNA was isolated from explant tissue cultured as described in Section 5.1.2. MMP-3 and -13 message was very low in control cultures but upon treatment by IL-1α there was a massive induction of mRNA levels, especially for MMP-3. This observed induction of both MMP-3 and -13 was seen to decrease after supplementation with U0126 (Figure 6.4). However, the expression of each TIMP (1-3) appears to be constitutive and there was no obvious change with either IL-1 or inhibitor treatment.

Monoclonal antibody, BC-14 is able to recognise the amino-terminal neoepitope sequence (FFGxx....) generated after MMP catabolism of aggrecan within the interglobular domain of the protein core (Caterson et al., 1995). This was used in Western blotting to investigate potential MMP cleavage of aggrecan but no positive bands i.e. no detectable aggrecan cleavage was found indicating that no MMP-generated catabolism of aggrecan was occurring (data not shown). However, this work was not attempted using a positive control for MMP-cleavage of aggrecan and, therefore, this experiment should be repeated to confirm that the BC-14 antibody is optimised and specifically recognising potential cleavage products of aggrecan.
6.3 Discussion

The aim of the work in this chapter was to investigate the signalling mechanisms involved in IL-1-induced activation of cartilage degradation and inflammation using the ERK-1 and -2 pathway inhibitor, U0126. Previous work has shown that IL-1 can induce the activation of MAPKs in chondrocytes and chondrosarcoma cells isolated from human and rabbit knees (Geng et al., 1996; Scherle et al., 1997; Mengshol et al., 2000) and the same kinases have been found in their phosphorylated form in rheumatoid synovial fibroblasts, activated in response to IL-1 (Schett et al., 2000). Our results show that U0126 supplementation of bovine chondrocyte explant and monolayer cultures leads to a decrease in IL-1-induced GAG release and aggrecanase activity, aggrecanase-1 (ADAMTS-4), MMP-3 and MMP-13 mRNA levels as well as, COX-2 message, protein and COX-2 activity (measured as PGE₂ production). As U0126 is a specific inhibitor of MEK-1 and -2, these results imply a role for the ERK pathway in IL-1 induced cartilage degradation and inflammation.

Several papers, from the same research group, have highlighted the ability of U0126 to inhibit MMP and aggrecanase production by inhibition of the ERK pathway (Liacini et al., 2002; Liacini et al., 2003; Sylvester et al., 2004). The group first reported a U0126 dose-dependent decrease in IL-1-induced MMP-3 and MMP-13 mRNA expression in bovine and human chondrocytes (Liacini et al., 2002), and next a down-regulation of TNF-stimulated MMP-13 by U0126 in human OA femoral head chondrocytes (Liacini et al., 2003), suggesting implication of the ERK pathway in cytokine-induced MMP gene expression. Recently the same group published evidence to suggest that U0126 was also able to inhibit IL-17-induced MMP-3, MMP-13 and ADAMTS-4 (aggrecanase-1) gene expression in bovine articular chondrocytes (Sylvester et al., 2004). The group’s work also indicates that other signal pathway inhibitors are able to reduce MMP expression and, hence, imply the need for cross talk between various pathways including the ERK, JNK and p38 kinase pathways, as well as NFκB and AP-1 transcription.
factor regulation. The combined results of these studies suggest common signalling pathways regulating MMP and ADAMTS-4 gene expression in cartilage metabolism. Our data also suggests that MMP-3, -13 and ADAMTS-4 (but not ADAMTS-5) gene expression is regulated by the ERK-1 and -2 pathways. Previous studies by Curtis et al., (2000; 2002) showed that n-3 PUFAs, but not n-6 PUFAs, were able to reduce the gene expression of IL-1-induced degradative enzymes such as MMP-3, -13 and ADAMTS-4 and -5 (aggrecanase-1 and -2, respectively) in bovine and human articular chondrocytes. This suggests a possible mechanism for the action of n-3 PUFAs in specifically targeting the ERK-1 and -2 pathways, preventing ERK activation by MEK, in the same manner as the commercial inhibitor U0126. However, another commercial ERK pathway inhibitor, PD98059 seemed to have no effect on aggrecanase activity in our preliminary studies (Chapter 5). The two inhibitors, U0126 and PD98059 each have distinct mechanisms of action. PD98059 binds only to, and inhibits the inactive form of MEK-1. However, U0126 is able to inhibit MEK-2 as well as MEK-1 and is capable of directly inhibiting activated MEK-1 and preventing endogenously active MEK-1 and -2 from phosphorylating and activating ERK-1 and -2 (Goueli et al., 1998) (see Figure 6.1). As PD98059 only prevents activation of ERK-1, it may, therefore, be the ERK-2 pathway that is the mechanism behind IL-1-induction of aggrecanase activity and, hence, it may be here that n-3 PUFAs are having their effects.

Danesch et al., (1994) previously reported that arachidonic acid (AA) strongly stimulates mRNA expression of the immediate early genes (IEGs) egr-1 and c-fos in a 3T3 fibroblast cell line. Subsequently, they discovered that blocking the conversion of AA into prostaglandins, by inhibiting cyclooxygenase, abolished the AA-induction of both IEGs. Inhibition of the lipoxygenase pathway had no effect, hence, they concluded that prostaglandin synthesis was necessary for the increase in egr-1 and c-fos mRNA. PGE$_2$ is the most abundant prostaglandin in
3T3 fibroblasts, and when added to the cells, PGE\textsubscript{2} stimulates egr-1 and c-fos gene expression in the same manner as AA (Danesch \textit{et al.}, 1994). The same group have since reported that n-3 PUFAs, specifically DHA and EPA, are able to decrease arachidonic acid (AA)-induced egr-1 and c-fos mRNA accumulation in the same 3T3 fibroblast cell line (Danesch \textit{et al.}, 1996).

Another study shows that U0126 is able to suppress c-fos mRNA and protein in tetradecanoylphorbol-13-acetate-induced fibroblasts (Favata \textit{et al.}, 1998). It is likely then that a similar mechanism is occurring in bovine and human chondrocytes. Here, our studies show that inhibition of the ERK pathway by U0126 is inhibiting COX mRNA expression, COX protein and PGE\textsubscript{2} production, which then may be regulating c-fos and egr-1 gene expression. Our preliminary array results also suggest that n-3 PUFA (EPA) supplementation may be able to down-regulate c-fos and egr-1 mRNA expression in IL-1-induced human OA chondrocytes. This result, therefore, compares well with the actions of U0126.

6.4 Chapter Summary

- Phosphorylated extracellular signal-regulated kinases (ERKs) -1 and -2 levels are decreased in the presence of specific ERK pathway inhibitor, U0126.
- IL-1 induced GAG release into the surrounding media of bovine articular cartilage explants was significantly decreased following addition of 25\textmu M U0126.
- IL-1 induced aggrecanase-1 (ADAMTS4) mRNA expression and aggrecanase enzyme activity were decreased after supplementation with U0126, in agreement with the observed release of GAG from the tissue into the media.
- There was no significant effect on aggrecanase-2 (ADAMTS-5) mRNA expression upon addition of U0126.
- MMP-3 and MMP-13 mRNA levels were increased in the presence of IL-1 and were reduced, in an apparently dose-dependent manner, following treatment with U0126.
• IL-1 inducible COX-2 message, protein, and its product, PGE₂, were all abrogated dose-dependently with U0126.

• In contrast, TIMPS-1, -2, -3, and aggrecan mRNA levels were unaffected by either IL-1α or U0126 treatment.

• Inhibition of the ERK pathway, by supplementation with U0126, resulted in a reduction of the IL-1 inducible degradative enzymes and inflammatory factors associated with osteoarthritis in a similar manner to the effects previously observed by n-3 polyunsaturated fatty acids (Curtis et al., 2000).
CHAPTER 7 - GENERAL DISCUSSION

Osteoarthritis is one of the most frequent diseases to affect the elderly of the Western world. The disease is associated with increased catabolism and the loss of the collagen and the major proteoglycan, aggrecan, resulting in a loss of articular cartilage as well as an increase in pro-inflammatory factors within synovial joints such as the knee.

The beneficial effects of cod liver oil on patients suffering from sore and inflamed joints have been published as far back as 1783 (Percival, 1783). Studies have also shown a lowered incidence of arthritis and musculoskeletal diseases in an Inuit population, which was attributed to their fishy diet, rich in n-3 PUFA (Kromann and Green, 1980). Previous work from within our laboratory has shown that n-3 PUFAs (including those found in fish oil), but not other fatty acids, have a beneficial effect on reducing the expression and activity of degradative enzymes (MMPs and ADAMTS [aggrecanases]) and inflammatory factors (cyclooxygenase-2, lipoxygenase-5, IL-1 and TNFα) which together cause the damage and destruction of cartilage seen in arthritis (Curtis et al., 2000; 2002). However, at present, the molecular mechanisms by which n-3 PUFA have their effects are still unknown. Therefore, the overall aim of this project was to investigate the effects of n-3 PUFAs on the expression of inflammatory factors at the proteomic level and to use specific pathway inhibitors to identify possible signalling pathways as potential targets for the actions of n-3 PUFAs.

In our initial studies (Chapter 3) we set out to investigate the effects of n-3 PUFA supplementation on IL-1-induced COX-2 expression, protein and activity (measured by PGE2 production), in normal bovine and human osteoarthritic chondrocytes. A well-established in vitro model of cartilage degradation was used (Hughes et al., 1998). The model uses chondrocyte monolayer cell cultures and explant cultures stimulated with catabolic agents (in this case IL-1) to induce a degradative phenotype. This same model was used in the previous investigations

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examining the effects of \( n-3 \) and \( n-6 \) PUFAs on the expression of various degradative enzymes and inflammatory factors, mentioned above. In this study we found that \( n-3 \) PUFAs (especially EPA) were able to abrogate increased COX-2 mRNA and protein expression induced upon IL-1 stimulation. EPA was also able to lower IL-1-induced PGE\(_2\) production and, hence, COX-2 activity, back to basal levels. These new results [as well as previous data (Curtis et al., 2000; 2002)] indicate that COX-2 protein, as well as other pro-inflammatory agents and degradative enzymes are decreased in the presence of \( n-3 \) PUFAs in both bovine and human models of arthritis and new studies have shown beneficial effects in canine (Curtis et al., 2004b) and feline (unpublished data) models, suggesting a potential for dietary supplementation in pet foods as a means of abrogating degenerative joint disease in these animals. \( n-3 \) PUFAs are found in abundance in fish oils such as cod liver oil and, hence, dietary supplementation may be potentially beneficial in regard to preventing the propagation of inflammation in diseases such as arthritis. Recent work within our group has focused on a pilot clinical trial on 25 patients scheduled for elective total knee replacement surgery for osteoarthritis. Half of the group were given cod liver oil capsules (2g/day) whilst the other half were given a mixture of edible oils in capsule form (a placebo, with the same calorific content) for 10-12 weeks prior to surgery. Cartilage, synovium and synovial fluid were all collected as waste products during surgery, whilst blood samples were taken both pre- and post-operation to analyse PUFA content (Local Ethical Approval was obtained). Early results show that 86\% of patients taking cod liver oil had a significant decrease in endogenous aggrecanase activity (compared with 26\% of placebo patients). 73\% had a decrease in endogenous collagenase (compared to 18\%) and 93\% had a decrease in aggrecanase mRNA expression. There was also a decrease in IL-1\( \alpha \) and TNF\( \alpha \) mRNA levels among the cod liver oil-taking patients (Curtis et al., 2004c). Interestingly, COX-2 levels were not reduced (as expected from early in vitro studies). However, this may have been
due to the fact that these patients were all at end stage OA where inflammation is at its highest and, thus, the dose of cod liver oil may be insufficient to effect COX-2.

Non-steroidal anti-inflammatory drugs (NSAIDs) are targets of cyclooxygenases and can control prostaglandin-induced inflammatory responses by inhibition of prostaglandin synthesis (Bauer and Marker-Hermann, 2003). The problems associated with NSAID use (e.g. gastrointestinal side effects) have been attributed to the inhibition of constitutive COX-1 (Dickman and Ellershaw 2004). Therefore, COX-2 selective inhibitors have been designed which have the beneficial effects on inflammation without removing the normal actions of COX-1 needed for normal homeostasis. As n-3 PUFA's have their effects on COX-2, but not on COX-1, fish oils such as cod liver oil could be cheap and easily accessible anti-inflammatory drugs in the treatment of osteoarthritis and potentially other inflammatory diseases such as rheumatoid arthritis, asthma and Crohn's disease.

The results described above, led us to investigate potential pathways associated with the effects of n-3 PUFA-supplementation on the IL-1-induced degradative enzymes and factors associated with cartilage destruction and inflammation propagation (Chapter 4). We used a simple array system (Human PathwayFinder-1 GEArray) to analyse the expression of several genes from several different signalling pathways. Human cartilage explants were obtained from osteoarthritic patients undergoing total knee replacement surgery. The explants were supplemented with or without n-3 PUFA's in the presence or absence of IL-1 prior to hybridisation to the array membrane. From this we identified potential signalling pathways implicated in the action of n-3 PUFA's on cartilage chondrocytes. These included the mitogen activated protein kinases (MAPKs), the NFκB transcription factor and it's pathway, and the anti-proliferation pathway.

Following identification of these potential signalling pathways, specific pathway inhibitors (PD98059, U0126, SB203580, curcumin and PDTC) were introduced into our bovine models of
arthritis to ascertain whether or not their inhibition profiles mimicked those seen with n-3 supplementation (Chapter 5). Inhibitors of the ERK, JNK, p38 and NFκB pathways (see Figure 7.1) were added in the absence or presence of IL-1 to bovine monolayer and explant cultures. Preliminary results identified three pathway inhibitors affecting endogenous aggrecanase activity and GAG release. These analyses indicated that supplementation with the ERK pathway inhibitor, U0126, and the NFκB inhibitor, PDTC, both resulted in a significant decrease of IL-1-induced aggrecanase activity and GAG release from the tissue, whilst SB203580, a p38 kinase inhibitor appeared to further induce activity and GAG release.

The NFκB transcription factor signalling pathway have previously been implicated in the production of inflammatory mediators such as iNOS and COX-2 upon cytokine induction. In fact, a large array of NFκB inducers (including cytokines, bacteria and viruses) and well over 150 target genes have been recognised (reviewed by Pahl, 1999). With regards to extracellular matrix genes, sequencing of the small leucine-rich proteoglycan, biglycan gene revealed the presence of an NFκB binding site in the 5' flanking region which could, potentially, be transcriptionally activated by NFκB (Ungefroren and Krull, 1996). Ongoing studies within our group have analysed the 5' non-coding region of the murine ADAMTS-4 (aggrecanase-1) gene. Sequence analysis has revealed four putative NFκB binding sites, three of which are conserved in humans. Early data shows that IL-1 can up-regulate ADAMTS-4 transcription in chondrocytes via NFκB binding to multiple binding sites located in the 5' non-coding region of this gene (Hughes et al., 2003). NFκB binding sites have also been found in rat and human cyclooxygenase-2 (Yamamoto et al., 1995), human cytosolic phospholipase A2 (Mori et al., 1994), human IL-1α (Mori and Prager, 1996) and human IL-1β (Hiscott et al., 1993) genes. Together this evidence suggests that the NFκB signalling pathway may be involved in the IL-1 induced degradation of cartilage and production of inflammatory mediators. Further work using the NFκB inhibitor, PDTC may elucidate whether n-3 PUFAs are affecting this pathway.

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Figure 7.1 A simplified representation of the ERK, JNK, P38 kinase and NFkB pathways with the sites of action of specific signal pathway inhibitors (PD98059, U0126, SB203580, curcumin and PDTG) highlighted. A possible target for n-3 PUFA action is also marked. Abbreviations: TAK-1, TGF-β activated kinase-1; IKK, IKB kinase.
specifically. In one study, the n-3 PUFA EPA has been reported to directly target NFκB activation in a dose-dependent manner in bovine chondrocytes, with suppression occurring immediately after IL-1 supplementation and, hence, the stimulation of inflammation (Longman et al., 2004).

In Chapter 6, experiments were carried out to analyse the effects of the ERK pathway inhibitor, U0126 on degradative enzymes and inflammatory factors associated with the onset of arthritis. Our results show that U0126 abrogates IL-1-induced GAG release, endogenous aggrecanase activity, ADAMTS-4 (but not ADAMTS-5) mRNA levels, MMP-3 and MMP-13 mRNA levels, COX-2 message, protein and PGE₂ production in a manner similar to that seen with n-3 PUFA supplementation of human and bovine chondrocyte cultures (see Figure 7.1). Figure 7.2 highlights the evidence for suggesting that U0126 and n-3 PUFAs both influence chondrocyte metabolism by a similar mechanism. This figure also highlights and summarises the novel findings described in this thesis.

An interesting result to note was the effect of U0126 on aggrecanase activity. U0126 decreased IL-1-induced aggrecanase cleavage of aggrecan, but not down to basal levels (see Figure 6.3). Further investigation into the gene expression of ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) revealed that only ADAMTS-4 was decreased upon U0126 supplementation (see Figure 6.4). An additional experiment that could determine whether U0126 is only affecting ADAMTS-4 cleavage of aggrecan, would be to use an antibody to block ADAMTS-5 activity or to use siRNA to knockout ADAMTS-5 activity. Under such conditions, it is expected that aggrecanase activity would be abrogated completely back to control-levels, upon addition of U0126.

Previous work has shown that collagenases (MMP-1, -8 and -13) and stromelysin (MMP-3) expression and activity is up-regulated in acute inflammation and inflammatory diseases (e.g. osteoarthritis) (reviewed in Vincenti and Brinckerhoff, 2002; Billinghurst et al., 1997; Mitchell

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**MMPs**

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**Mitogenic Pathway**

- Decreased levels after IL-1-induction in bovine and/or human chondrocyte cultures (Results from this work)
- Decreased levels after IL-1-induction in bovine and/or human chondrocyte cultures (Curtis et al., 2000; 2002)

**Figure 7.2** A comparison between the observed effects of U0126 and n-3 PUFA (EPA) supplementation. Abbreviations: PUFA, polyunsaturated fatty acid; PGE₂, prostaglandin E₂; GAG, glycosaminoglycan; MMP, matrix metalloproteinase; ADAMTS-4, aggrecanase-1; ADAMTS-5, aggrecanase-2. * research was not carried out in chondrocytes.

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et al., 1996) as well as in experimental models of cartilage degradation (Bluteau et al., 2001; Curtis et al., 2002; Chu et al., 2002; Hui et al., 2003). There is also evidence to suggest that n-3 PUFAs can reduce cytokine-induced collagenase expression in articular cartilage chondrocytes (Curtis et al., 2002). A monoclonal antibody, 9A4, was designed specific for the C-terminal neoepitope sequence generated by collagenase cleavage of type II collagen (Gly-Pro-Pro-Gly-Pro-Gln-Gly-COOH) (Otterness et al., 1999). Data shown in Chapter 6 shows that IL-1-induced MMP-3 and -13 gene expression was reduced upon addition of U0126. In future studies, it would be interesting, therefore, to investigate the effects of U0126 on endogenous collagenase activity either by Western blotting or immunohistochemistry with the 9A4 monoclonal antibody to recognise cleavage products produced by collagenase. Thus, we would expect these experiments to show abrogation of collagenase activity at the proteomic level.

During our studies analysis relating to mRNA expression was carried out using reverse transcriptase PCR (RT-PCR) using equal amounts of RNA for each reaction and normalising to the expression levels of a constitutive housekeeping gene, GAPDH. However, this method is only semi-quantitative. Real-time quantitative PCR is a very powerful and sensitive method used to quantify mRNA expression levels of genes, which are often very low in the tissues under investigation (e.g. cytokines). The latter PCR method is very accurate and sensitive, allows a high throughput, and can be performed on very small samples (Giulietti et al., 2001). Therefore, to obtain more quantitative and reliable results about the effects of n-3 PUFAs or selective inhibitors on cytokine expression it may be a good idea to use quantitative PCR in future work.

Taken together, the combined results presented in this thesis suggest a possible role for the extracellular signal-regulated (ERK) pathway and the NFκB pathway in IL-1-induced degradation and inflammation of articular cartilage. Our results also suggest that inhibition of the ERK pathway and/or the NFκB pathway may be possible mechanisms by which n-3 PUFAs are able to specifically inhibit IL-1-induced degradative enzyme and inflammatory factor mRNA
and protein expression. Both pathways could, therefore, be possible targets for therapeutic intervention of inflammatory diseases such as osteoarthritis.
CHAPTER 8 - REFERENCES


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