The Effects of Anti-inflammatory Drugs on Cartilage Breakdown and their Mechanism of Action on Chondrocytes

Rachel Louisa Lakey

A thesis is submitted to the
University of Northumbria at Newcastle
for the degree of Doctor of Philosophy

Research undertaken in the School of Applied Sciences
and in collaboration with the Musculoskeletal Research Group, Newcastle University.

June 2008

Dedicated

in the loving memory

of

Edwin Basil Rose

(1918 – 1978)

For Grandpa

Abstract

The progressive loss of cartilage matrix is a major characteristic of arthritic disease, ultimately leading to a loss of joint function. A number of therapeutics are used in the treatment of arthritic disease, with non-steroidal anti-inflammatory drugs used to treat the pain and inflammation seen in osteoarthritis and rheumatoid arthritis, whilst disease modifying anti-rheumatic drugs are used to slow disease progression. However it is not fully understood if and how many of these drugs effect the disease processes in arthritis. The objective of this study is to look at a number of therapeutics and investigate their effect on the breakdown of proteoglycan and collagen, and on the expression of a number of key degradation enzymes, whilst trying to identify the possible mechanisms used by the drugs.

Using the bovine nasal cartilage explant model IL-1 + OSM were used to stimulate the release of proteoglycan and collagen from the cartilage. Interleukin-1 (IL-1) and oncostatin M (OSM) together promote the degradation of cartilage by up regulating and activating MMPs that are found within the diseased joint. Treatment of the resorbing cartilage with indomethacin, indomethacin heptyl ester, simvastatin, mevastatin, pravastatin and sulfasalazine produced a variation of findings with many resulting in the inhibition of cartilage degradation. The stimulation of human articular chondrocytes with IL-1 + OSM caused a significant up regulation of MMPs by the cells at both a gene and protein level when measured by TaqMan PCR and ELISA respectively. Again treatment of the stimulated chondrocytes with a number of the drugs showed a significant reduction in the expression of key cartilage degrading enzymes.

The investigation of signalling mechanisms affected by one specific drug, namely sulfasalazine by immunoblotting and signalling microarray, showed some interesting results not previously documented. Sulfasalazine is well known to inhibit NF-κB activation by blocking the degradation of IκB to the proteosome and data in this study supports these finding. However, study of the MAPK pathway showed that sulfasalazine appears to be able to block the signalling cascade which ultimately leads to AP-1 activation in chondrocytes stimulated with IL-1 + OSM.

This study has identified possible chondroprotective properties in a number of the drugs which were screened and whilst the exact mechanisms behind these events still require further investigation, the results highlight the potential of these drugs in being used to

prevent further cartilage degradation in arthritis and thus further delaying the possible need for joint replacement operations.

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Abbreviations

5-ASA	-	5-aminosalicylic acid
ADAMTS	-	a disintergrin and metalloproteinase with thrombospondin motif
AP-1	-	activator protein-1
APMA	-	ρ-amino phenyl-mercuric acetate
APS	-	ammonium persulphate
ATF	-	activating transcription factor
BC	-	bacterial collagenase
BNC	-	bovine nasal cartilage
BSA	-	bovine serum albumin
cDNA	-	complementary DNA
C	-	cysteine
CA	-	cysteine array
CCA	-	cacodylate
C/EBP	-	CAAT/enhancer-binding protein
COX	-	cyclooxygenase
CRD	-	cysteine rich domaine
CS	-	chondroitin sulphate
Cyt	-	cytoplasmic tail
DAB	-	3, 3'-diaminobenzidine
d.H20	-	distilled water
DMARD	-	disease-modifying anti-rheumatic drug
DMB	-	1,9-dimethylmethylene blue
DMEM	-	Dulbecco's modification of Eagle's medium
DMSO	-	dimethylsuphoxide
DNA	-	deoxyribonucleic acid
DPBS	-	Dulbecco's phosphate buffered saline
DTT	-	DL-dithiothreitol
ECL	-	electrophoresis-chemiluminescence
ECM	-	extracellular matrix
EDTA	-	ethylenediaminetetraacetic acid
ELISA	-	enzyme-linked immunosorbent assay

ERK	-	extracellular signal-regulated kinase
FCS	-	foetal calf serum
GAG	-	glycosaminoglycan
GAPDH	-	glyceraldehyde-3-phosphate dehydrogenase
Gly	-	glycine
HAC	-	human articular chondrocytes
HEPES	-	*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid
HIF	-	hypoxia inducible factor
HMG-CoA	-	hydroxymethylglutaryl-Coenzyme A
Hpx	-	hemopexin
HRP	-	horse-radish peroxidise
IFN	-	interferon
Ig	-	immunoglobular
IGF	-	insulin growth factor
IL	-	interleukin
ILR	-	interleukin receptor
IND	-	indomethacin
INDHE	-	indomethacin heptyl ester
JNK	-	c-Jun N-terminal kinase
MAPK	-	mitogen-activated protein kinase
MKK	-	MAPK kinase
MMP	-	matrix metalloproteinase
mRNA	-	messenger RNA
MT-MMP	-	membrane type-MMP
MTX	-	methotrexate
NF-κB	-	nuclear factor κB
NSAID	-	non-steroidal anti-inflammatory drug
OA	-	osteoarthritis
OPD	-	o-phenylenediamine
OSM	-	oncostatin-M
PCR	-	polymerase chain reaction
PGE	-	prostaglandin E
PMSF	-	phenylmethanesulphonylfluoride

PVDF	-	polyvinyl difluoride
RA	-	rheumatoid arthritis
RHD	-	rel homology domain
RNA	-	ribonucleic acid
RQ	-	relative quantification
RT	-	room temperature
RT-PCR	-	reverse transcriptase PCR
SDS	-	sodium dodecyl sulphate
SDS-PAGE	-	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	-	systemic lupus erythrematosus
SP	-	sulfapyridine
SSZ	-	sulfasalazine
TBS	-	tris buffered saline
TEMED	-	NNN'N'-tetramethylethylene-diamine
TGF	-	transforming growth factor
TIMP	-	tissue inhibitor of metalloproteinases
TNF	-	tumor necrosis factor

Acknowledgements

Firstly I would like to thank my supervisors, Tim Cawston, Mark Daniels and Chris Baldwin, for their help, support and patience through out my PhD, and also to the ARC and School of Clinical Medical Sciences for their financial support. I feel I have come along way since this all started and I hope that what I've learnt will help me in years to come. Thanks also to Jon Catterall, not only for keeping me laughing and smiling throughout and giving me someone to nag when our bay was a mess or for stealing my pipettes, but also for those drunken Friday nights in the North terrace where the discussions we had helped me plan and often see the light!

Thanks also have to go to members of the department past and present, particular thanks go to "the tech team" Tim, Debbie, Julie and Janet not only for their encouragement and support but also for being there when I needed to have a good moan. Thanks also go to all the "tunnel dwellers", thanks for being a laugh and keeping everything real and there are a few individuals who deserve a special mention: Ghada for your friendship and support not only on a professional level but also a personal one too. To Helen, Marina, Christine, Jenny and Amy for the laughs and gossips, and not to forget Matt J for all those rugby discussions which helped keep me sane. Thanks also go to the Mann Clan girls Silvia and Shilu for the lunch time laughter that always helped lift the mood.

Away from work I would like to thank my friends, of which there are too many to mention personally, but your support and laughter has kept me going, Thank you! Special thanks also go to my brothers Richard and Mark for beating me up as a child but making me a better person for it! (Only joking.....!) And also my gran for her love and support, and to Ken and Joyce for your love, interest and support.

Two people require a personal mention. Firstly, Kenny your love, support and strength has kept me going when the chips were down and I couldn't think of anyone else I rather spend my life with – I love you loads!

Lastly, thanks to my Mum and probably my best friend, without your constant love and support none of this would have been possible, you have made me the person I am today and for that I thank you. I love you loads and all I hope is that I have made you proud!

Declaration

This these is based on research preformed in the Musculoskeletal Research group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK for the School of Applied Sciences, University of Northumbria, Newcastle upon Tyne, UK. Except for commonly held concepts, and where specific reference is made to other work, the content of the thesis is original. No part of this thesis has been submitted for the award of any other degree.

List of Publications

T. Z. Sukkar, J. M. Thomason, T. E. Cawston, **R. Lakey**, D. Jones, J. Catterall, R. A. Seymour. "Gingival fibroblasts grown from cyclosporin-treated patients show a reduced production of matrix metalloproteinase-1 (MMP-1) compared with normal gingival fibroblasts, and cyclosporin down-regulates the production of MMP-1 stimulated by pro-inflammatory cytokines" J Periodontal Res. 2007 Dec;42(6):580-8.

HE Barksby, JM Milner, AM Patterson, NJ Peake, W Hui, T Robson, **R Lakey**, J Middleton, TE Cawston, CD Richards, AD Rowan. "Matrix metalloproteinase 10 promotion of collagenolysis via procollagenase activation: implications for cartilage degradation in arthritis". Arthritis Rheum. 2006 Oct;54(10):3244-53.

DA Young, **RL Lakey**, CJ Pennington, D Jones, L Kevorkian, DR Edwards, TE Cawston, IM Clark. "Histone deacetylase inhibitors modulate metalloproteinase gene expression in chondrocytes and block cartilage resorption". Arthritis Res Ther. 2005;7(3):R503-12. Epub 2005 Feb 22.

Rachel L Lakey, Tim E Cawston "Sulfasalazine blocks the release of proteoglycan and collagen from cytokine stimulated cartilage and down regulates the increase of matrix metalloproteinases". (Submitted to Arthritis and Rheumatism)

Gary J Litherland, Craig Dixon, **Rachel L Lakey**, Debra Jones, David A Young, Tim E Cawston, Andrew D Rowan. "Synergistic collagenase expression and cartilage collagenolysis are phosphatidylinositol 3'-kinase/Akt signalling-dependent". J Biol Chem 2008, In Press.

Rachel L Lakey, Debra Jones, Jonathan B Catterall, Tim E Cawston. "Effect of Statins on cytokine induced cartilage degradation". (Submitted to Arthritis and Rheumatism)

RL Lakey, TG Morgan, AD Rowan, TE Cawston, CMU Hilkens. "A novel paradigm for dendritic cells as effector cells in cartilage destruction" (Submitted to Arthritis and Rheumatism)

Oral Presentations

Oral presentation for the Annual Northern and Yorkshire Rheumatology meeting (2007)

Abstracts

Rachel L Lakey and Tim E Cawston. "Sulfasalazine blocks the release of proteoglycan and collagen from cytokine stimulated cartilage and down regulates matrix metalloproteinases". (2007) Annual Northern and Yorkshire Rheumatology meeting.

Rachel L Lakey, Debra Jones, Jonathan B. Catterall, Tim E. Cawston. "Mevastatin and Simvastatin block collagen release from bovine cartilage and downregulates MMP-13 from human articular chondrocytes". (2006). Rheumatology. Vol45:S1.

JB Catterall, **R Lakey**, D Jones, TE Cawston. "Cyclosporin inhibits interleukin 1 and oncostatin M induced cartilage breakdown." (2006). Rheumatology. Vol45:S1

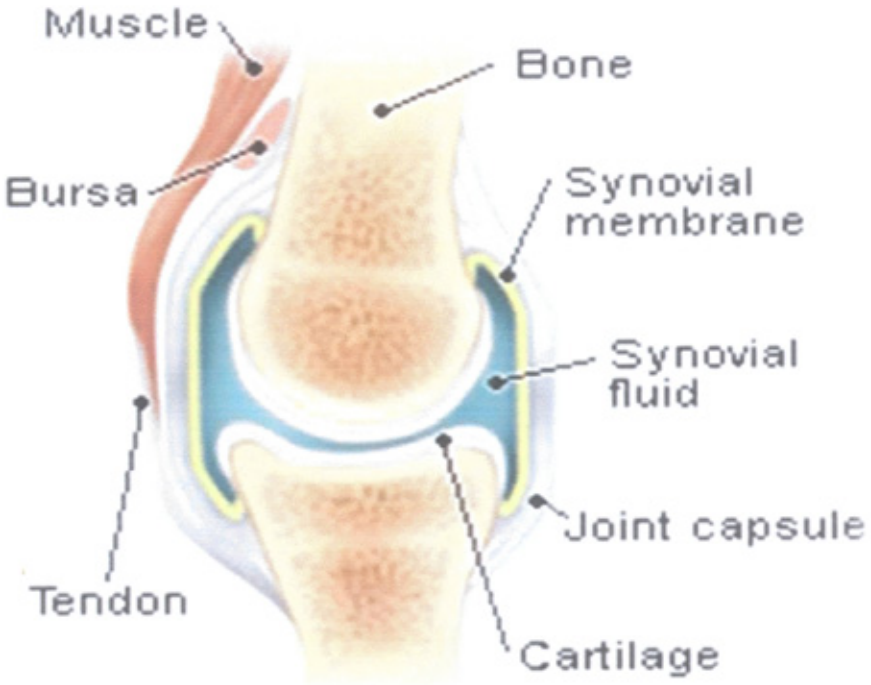
CHAPTER 1: Introduction

1.1 THE HUMAN ARTICULAR JOINT

1.1.1 Basic structure and function of the joint

Articular joints allow free movement of the associated skeletal bones and acts as a shock absorber for compressive loads generated by normal bodily function. It is comprised of seven major components, with each having a crucial role in normal joint function (figure 1.1). The skeletal bone provides the body with structural stability, whilst cartilage, discussed in section 1.1.2, allows friction-free movement to the joint and has shock distributing and absorbing properties. Synovial fluid synthesised by synovial cells in the joint lining supplies the cartilage with nutrients and provides lubrication for the smooth articular surfaces. The fibrous joint lining, with associated highly vascular synovial tissue, produces and maintains homeostasis of the synovial fluid and the integrity of the fluid capsule. A network of fibrous ligaments provide the joint with stability which holds the articulating surfaces in place and in turn works to prevent inappropriate and potentially damaging joint movement.

Figure 1.1 The normal human joint.



1.1.2 Articular Cartilage

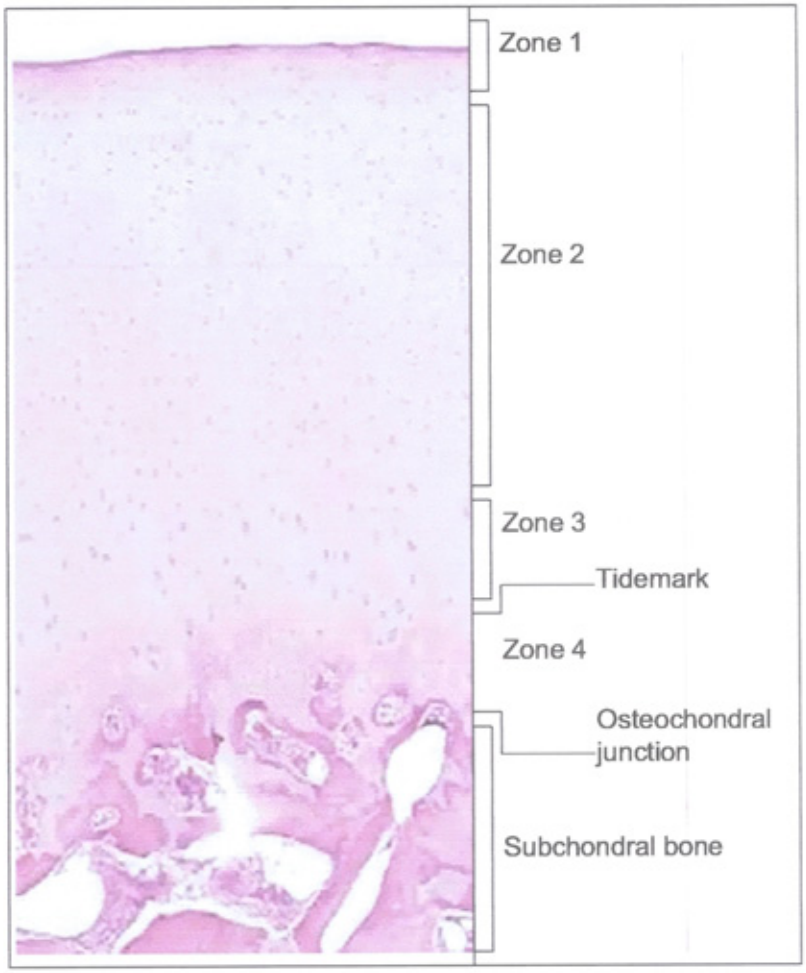
Connective tissues are vital to maintain the structure and function of a healthy body. One key connective tissue is articular cartilage which is found within synovial joints, which covers the ends of long bones. By working with synovial fluid within the joint it provides a smooth lubricated surface allowing the free and smooth movement of the joint with little friction occurring between articulating surfaces, whilst also protecting bones from abrasion. During rheumatic disease (section 1.2), it is the progressive destruction of the cartilage matrix and the underlying bone which ultimately leads to a loss of joint function.

In normal articular cartilage the structural organisation, gives it tensile strength and elasticity allowing it to absorb and distribute the stresses of biomechanical loading (Huber et al. 2000). The lack of blood vessels, lymphatic vessels and nerves within the articular cartilage, coupled with a lack of a basement membrane makes it a unique connective tissue (Kuettner, 1992). Chondrocytes are the only cell type found within articular cartilage (section 1.1.3). When compared to tissues such as muscle or bone, cartilage has a low cell density, which results in a low metabolic activity, causing it to be less responsive to changes in loading or injury (Poole et al, 2001).

Cartilage is made up of two principle components: the tissue fluid and the structural framework of macromolecules which form a matrix giving the tissue its form and stability. The structural macromolecules of the cartilage which consist of collagens (section 1.1.4), proteoglycans (section 1.1.5) and non-collagenous proteins contribute between 20 – 40% of the wet weight of the tissue (Buckwalter and Mankin, 1997), whilst water contributes up to 80% of the wet weight of articular cartilage and is tightly bound within the extracellular matrix (ECM). The material strength and biological properties of articular cartilage depends highly upon the unique and complex 3D structure of collagen fibres which provide the tensile strength and are essential for maintaining tissue volume and shape. Highly hydrated negatively charged proteoglycans allow the cartilage to undergo reversible deformation and to resist compression while allowing equal distribution of load to weight bearing joints (Hardingham and Fosang, 1992). Normal cartilage turnover is viewed as a balance between the degradation and synthesis of the constituent macromolecules within the cartilage matrix. The destruction of the tissue is achieved when degradation exceeds synthesis and an alteration in the steady state affects the integrity of the cartilage (Huber et al, 2000; Shum et al, 2002; Eyre et al, 2006).

Articular cartilage consists of four distinct zones or horizontal layers (Figure 1.2) which are distributed through the cartilage from the surface to the subchondral bone. The zones are distinguished by chondrocyte size, shape and distribution, biochemical composition and arrangement of macromolecules (Buckwalter et al 1997(a); Poole, 1997).

Figure 1.2 Zones of articular cartilage. Courtesy of Dr Angela M. Patterson (see text for details).



Zone 1 (superficial zone) lies adjacent to the joint cavity and is the thinnest layer of articular cartilage. There are two collagen layers found within this zone, the first layer (lamina splendens) is deficient of cells and is made up of a sheet of fine fibrils which lie parallel to the articular surface. The second layer consists of flattened chondrocytes, a high water concentration, and low proteoglycan content and is densely packed with collagen

fibrils. The mechanical properties of the tissue are determined by the superficial zone. Dense layers of collagen fibrils which lay perpendicular to the articular surface act as a barrier to the passage of large molecules such as antibodies from synovial fluid to cartilage (Huber et al, 2000). Zone 2 (transitional and middle zone), is made up of rounded chondrocytes that synthesise a matrix that contains large-diameter collagen fibrils organised in a random arrangement. Proteoglycan content within this zone is high, with lower concentrations of water and collagen than in the superficial zone. In Zone 3 (radical zone) the chondrocytes are rounded and grouped in columns. The water content within the radical zone is low, with high proteoglycan content and a minimal collagen content, however the fibres have the largest diameter. A tide mark separates the deep zone from the calcified zone representing bands of fibrils. The calcified zone (zone 4) is made up of a thin layer of calcified cartilage that contacts the underlying subchondral bone. In this zone, the chondrocytes are rounded and low in volume, and located in uncalcified lacunae. Collagen fibres are arranged perpendicular to the articular cartilage and anchored in a calcified matrix, with no proteoglycans present in this zone. The subchondral bone plate (or osteochondral junction) separates the calcified layer from the subchondral bone. The subchondral bone works as a shock absorber and protects the cartilage against the damage caused by excessive loads (Buckwalter et al, 1997(a); Poole et al, 2001).

1.1.3 Chondrocytes

Chondrocytes are the only cells of normal adult articular cartilage that maintain the cartilage matrix under normal conditions. They are highly specialised mesenchymal cells which are considered to be terminally differentiated cells. Accounting for between 2 – 5% of the tissue volume, chondrocytes are relatively metabolically inactive due to the absence of a vascular supply in the tissue. The mature chondrocyte is spherical in shape and is able to synthesis all components of the ECM, however chondrocytes are known to vary in size, shape and metabolic activity depending on their zone of residence. The interactions which occur between chondrocytes and the ECM are highly important in order to regulate the development, maintenance and the repair of the ECM, which in turn protects the chondrocytes from damage and allows them to maintain their shape and phenotype (Muir, 1995; Goldring, 2000).

The articular surface requires constant maintenance with the continual renewal of matrix components. Chondrocytes are responsible for the synthesis of macromolecules, before organising and depositing them into a complex framework. Chondrocytes structurally

have short cilia which extend from the cell into the matrix, allowing the chondrocytes to sense any mechanical changes within the matrix. This may allow the chondrocyte to detect both the degradation of macromolecules and any change in the mechanical loads on the articular cartilage (Buckwalter et al, 1997(a)). Under physiological conditions, chondrocytes are able to preserve an active membrane transport system by exchanging cations such as Na^+, K^+, Ca^{2+} and H^+, which fluctuate in concentration depending upon load and changes in the composition of the cartilage matrix (Wilkins et al, 2000). Chondrocyte metabolism operates under low oxygen conditions within the cartilage matrix, where oxygen levels range from 10% at the surface to below 1% in the deep zones. During physiological hypoxia, the hypoxia inducible factor-1α (HIF-1α) acts as a survival mechanism for the chondrocyte and gives it the capacity to react to a change in its environment (Goldring, 2006).

Chondrocytes are surrounded by a narrow pericellular layer which is confined and enclosed within a fibrillar pericellular space, providing hydrodynamic protection for chondrocytes during loading. Within this pericellular environment high concentrations of sulphated proteoglycans can be found, along with a number of key cartilage matrix proteins (Table 1.1). CD44-like surface receptors which are found on the chondrocyte, allow interaction with hyaluronic acid to take place allowing proteoglycans to bind, via hyaluronic acid to the cell (Knudson et al, 1993). The chondrocyte in partnership with the pericellular microenvironment is commonly named the chondron (Poole, 1997). Once cartilage matrix is formed within the adult, chondrocytes are able to maintain a low rate of turnover of these matrix proteins, however there are regional differences in the remodelling of chondrocytes with matrix turnover being faster in the immediate pericellular zones (Goldring, 2006).

Chondrocytes are able to draw their nutrition from the synovial fluid, synthesised by the fibroblasts which are present in the synovial membrane. Synovial fluid is a plasma ultra filtrate produced from synovium and consists of water and nutrients such as glucose and glucose-derived sugars such as glucosamine sulphate and vitamin C. The nutrients reach the cell by diffusing from the synovium through the synovial fluid into the cartilage, where hyaluronic acid gives unique properties playing a key role in the development, maintenance, repair, and remodelling of cartilage (Mobasheri et al, 2002).

Table 1.1 Cartilage matrix proteins. Taken from Goldring, 2001.

Collagens
Type II
Type IX
Type XI
Type VI
Type X (growth plate)
Type XII, XIV
Proteoglycans
Aggrecan
Biglycan
Decorin
Fibromodulin
Lumican
Non-collagenous proteins
Cartilage oligomeric matrix protein
Cartilage matrix protein (matrilin)
Anchorin II
Tenascin
Thrombomodulin
Proline/arginine-rich end leucine-rich repeat protein.
Chondroadherin
Fibronectin
Membrane proteins
Syndecan
CD44
Integrins (α1, 2, 3, 5, 6, 10; β1, 3, 5)

During the aging process there is a progressive reduction in chondrocyte density and matrix synthesis, leading to a decrease in synthetic activity. It is thought that mechanical disruption of the chondrocyte-matrix associations may lead to an alteration in the metabolic responses of the chondrocyte (Guilak et al, 2004). In degenerative diseases such as osteoarthritis (OA), the behaviour of chondrocytes is reflected in the appearance of fibrillations, matrix depletion, cell clusters and changes in the quantity, distribution or composition of matrix proteins (Pritzker et al, 2006). The effect of mechanical loading is also thought to be responsible for the destruction of the cartilage matrix by causing an

increased regulation of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF-α), which lead to the progressive loss of articular cartilage (figure 1.2). The major events in the pathogenesis is localised with in the cartilage itself, and there is increasing evidence that the chondrocyte itself takes part in this destructive process by responding to catabolic cytokine release from other joint tissues, but also by producing the cytokines themselves (Goldring and Berenbaum, 2004; Goldring and Goldring, 2004). Unfortunately a failure to restore the cartilage tissue leads to a decline in chondrocytic response and death by apoptosis, and therefore leads to a deterioration of the ECM which is no longer adequately maintained.

1.1.4 Collagens

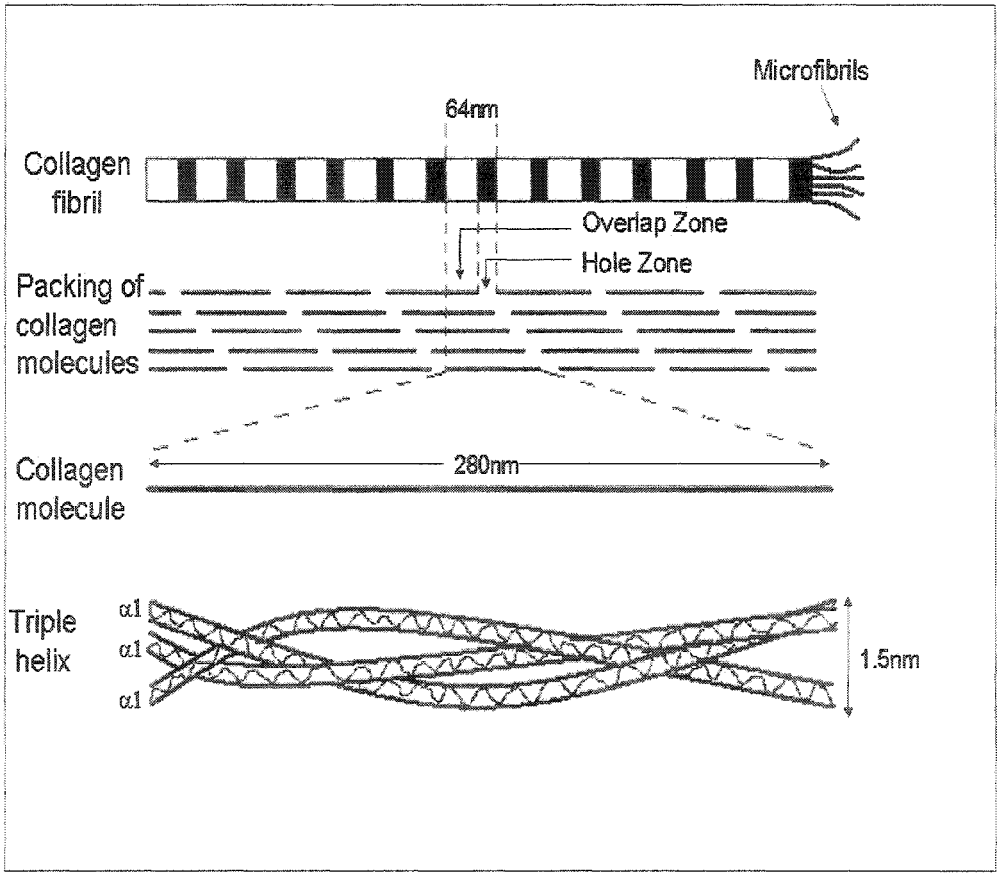
Collagen is the most abundant protein in the mammalian body, with ~30% of all protein within the human body being collagen. Vertebrates have at least 27 collagen types with 42 distinct α chains in total, and more than 20 additional proteins having collagen-like domains. Some collagens have a limited tissue distribution, for instance types II, IX and XI are found exclusively within cartilage and type X which is only found in hypertrophic cartilage. Collagen Type IV is a major structural component of basement membranes, whilst collagen type VII is found in anchoring fibrils for the basement membrane (Myllyharju and Kivirikko, 2004).

The primary structure of collagen molecules consists of three polypeptide chains (α-chains) and contains at least one domain composed of repeating Gly-X-Y sequences in each constituent chain. The three α chains are coiled into a left-handed helix and are wound around a common axis to form a right-handed triple helix, with the final structure being a rope-like rod. Glycine, the smallest amino acid is placed at every third residue and is vital to allow packing of the tightly coiled structure. Any amino acid other than glycine can take the X and Y position, however proline and 4-hydroxyproline are frequently (~25%) found in the X and Y positions respectively. The 4-hydroxyprolines play a key role in the stability of the triple helix, by not only stabilising the polypeptide chains but also by hydrogen bonds and water bridges which require the presence of 4-hydroxyproline (Brodsky and Ramshaw, 1997).

Type II collagen is the single most copious protein in normal articular cartilage, comprising of approximately 90% of total collagen in the cartilage matrix. It is a fibrillar collagen with three identical α-chains (figure 1.3) which interacts with other cartilage

specific collagens collagen type IX and XI forming a copolymer. Collagen IX molecules adorn the surface of type II collagen fibrils whilst collagen XI molecules are predominantly cross-linked to each other and are believed to form a template which constrains the lateral growth of collagen II (Eyre et al, 2006).

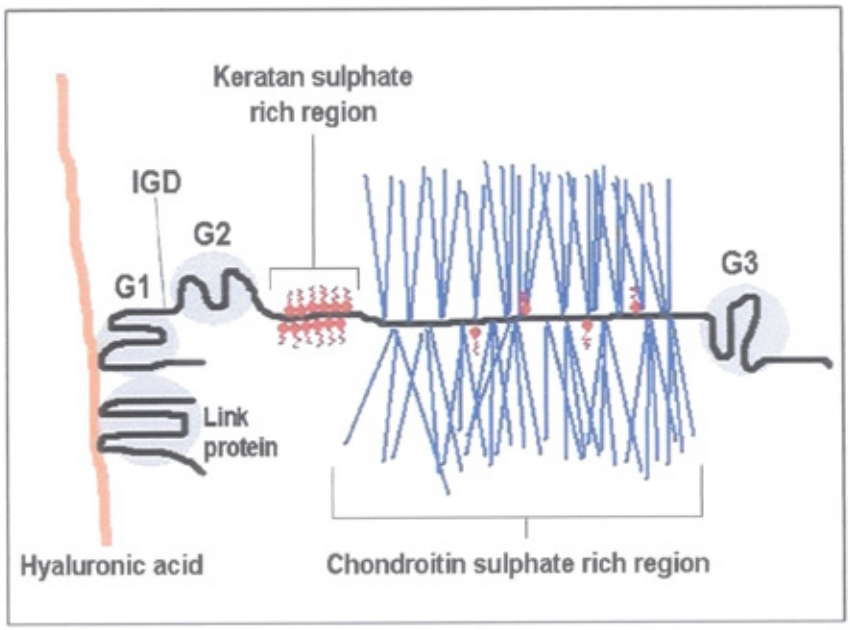
Figure 1.3 Structure and organisation of collagen



1.1.5 Proteoglycan

Proteoglycan is the second major component of articular cartilage. Existing as a cartilage-specific aggrecan, it associates with hyaluronan and a small glycoprotein, link protein to form aggregates. The aggregates have a high fixed negative charge due to a large number of polyanionic glycosaminoglycan chains on the aggrecan, thus allowing hydration of the tissue providing cartilage with a high water content of up to 80 % wet weight. This in turn acts as a space-filling gel which is responsible for the compressive resilience of articular cartilage during joint loading (Dudhia, 2005).

Figure 1.4 Structure of aggrecan. Aggrecan consists of three globular domains (G1, G2 and G3) separated by linear portions of polypeptide. The interglobular domain between G1 and G2 is particularly susceptible to proteolytic cleavage. The region between G2 and G3 binds highly sulphated glycosaminoglycans. Aggrecan binds to hyaluronic acid via the G1 domain and the binding is enhanced by link protein.



Aggrecan is the predominant form of cartilage proteoglycan (approximately 90% of the total). It is the core protein which consists of three globular domains, G1, G2 and G3, and two glycosaminoglycan-attachment domains, keratin sulphate (KS) and chondroitin sulphate (CS) domains (figure1.4). The G1 and G2 domains are found at the N-terminus of the core protein, and these are separated by a rod-shaped domain known as the interglobular domain (IGD). Located between the G2 and G3 domains, is a long extended region, the glycosaminoglycan (GAG) attachment region. This region is divided into three adjacent domains which are responsible for the attachment of KS (KS domain) and CS (CS1 and CS2 domains) (Roughley et al, 2006; Watanabe et al, 1998).

In addition to aggrecan, at least three other proteoglycans have been identified in articular cartilage (heparan sulphate, keratan sulphate and chondroitin sulphate). These are smaller and non-aggregating and are believed to play a role in the organisation of collagen molecules and may even influence chondrocytes function (Hedbom and Heinegard, 1993).

1.2 OSTEOARTHRITIS, RHEUMATOID ARTHRITIS AND THEIR TREATMENT

1.2.1 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and is known to affect millions of people all over the world. It is known to affect about 60% of men and 70% of women above the age of 65. It is a heterogeneous and multi-factorial disease which is characterised by the progressive loss of articular cartilage, but it also affects the entire joint such as the synovial membrane, joint capsule, ligaments, periarticular muscles and tendons, and the subchondral bone (figure 1.5) (Goldring, 2006). The initiation and pathogenesis of the disease can be affected by a number of factors which include altered mechanical loading and previous trauma or injury to the joint (Blumenkrantz et al, 2007). Symptoms include pain, stiffness and the impairment of joint motion, which in turn ultimately leads to disability and the need for joint replacement. Primary OA is characterised by late onset and has no obvious cause, whereas secondary OA has an early onset and has an identifiable cause such as injury or a developmental abnormality. OA can occur in any joint, but is most commonly found in the hand, knee, hip and foot. Pathological changes can be observed radiologically as a loss of joint space, subchondral bone sclerosis and the presence of osteophytes (bone spurs) located at joint margins (Goldring, 2006).

The chondrocytes (previously described in section 1.1.3) are of major clinical importance in the context of the pathogenesis of disease of OA, resulting from a failure to maintain the balance between synthesis and degradation of the extracellular matrix (ECM). The induction in the production of proteinases such as metalloproteinases (section 1.3.3) and A Disintegrin And Metalloproteinase with ThromboSpondin (ADAMTS) (section 1.3.5) by the chondrocytes and bone cells has an established association with cartilage destruction.

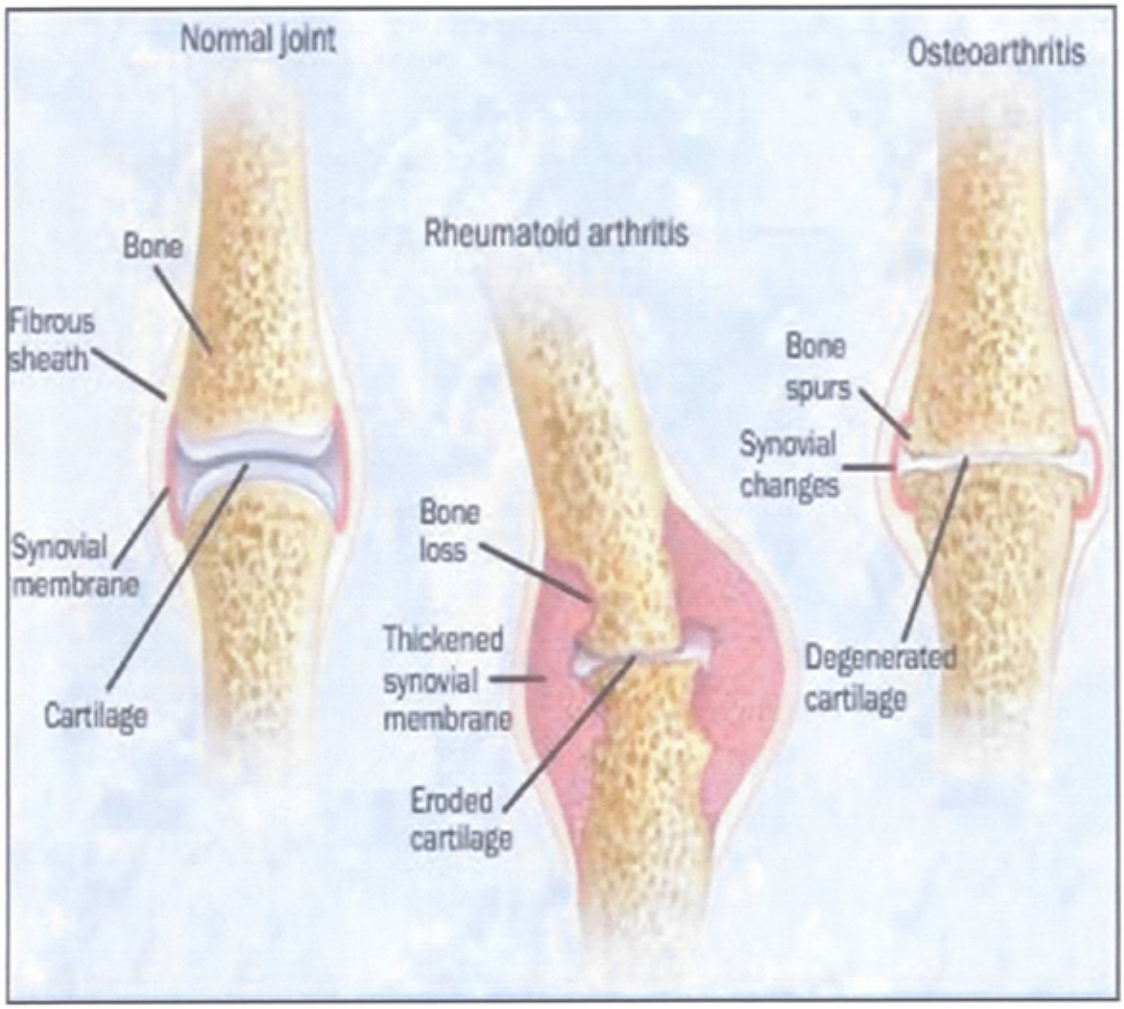
1.2.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) in a chronic connective tissue disease associated with bone and cartilage destruction. It is characterised by non-specific, usually symmetrical inflammation of the peripheral joints, which potentially results in the destruction of articular and periarticular joints and structures (Hassan et al, 2001). Studies have established that joint destruction occurs mainly when the pannus, a tumor-like proliferation of rheumatoid synovial tissue, is in contact with the intraarticular cartilage and bone (figure 1.5) (Woolley

et al, 1997). The pannus invades the superficial layers of the joint cartilage. Within the pannus/cartilage interface, synovial fibroblasts, macrophages, and neutrophils can be found, but the exact mechanism of these cells on the underlying cartilage destruction remains unclear. Chondrocytes are also involved, but the increase seen in chondrocytes metabolic rate is minimal compared to that seen in OA (Rannou et al, 2006).

Rheumatoid arthritis affects people throughout the world, however more severe cases are found in northern Europe. In Britain alone, more than 350,000 people have rheumatoid arthritis and it can affect people of any age, but the most common age for the disease to start is between 40 and 50. About three times as many women as men are affected. The breakdown of cartilage in RA is mediated by proteolytic enzymes which are regulated by mediators such as cytokines, growth factors, prostaglandins and matrix breakdown products.

Figure 1.5 Pathological manifestations in osteoarthritis and rheumatoid.



1.2.3 Non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to relieve pain and inflammation in patients with osteoarthritis and rheumatoid arthritis. NSAIDs can be divided into two groups, the traditional non-selective types and the selective cyclooyxgenase-2 (COX-2) inhibitors, and both types are effective anti-inflammatory and analgesic agents used in a wide range of acute and chronic medical conditions; examples include aspirin, ibuprofen, and indomethacin. NSAIDs are freely available and both a cheap and convenient form of treatment. However, both forms of NSAIDs are known to cause adverse effects in patients such as myocardial infarction, strokes and increased mortality in patients with and without pre-existing cardiovascular disease (Chin, 2007). Until recently these side effects were not commonly known, however the recent Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (Bombardier et al, 2000) have brought the contraindications of the drugs very much into the public eye.

1.2.3.1 Indomethacin

Indomethacin is an NSAID which is known to block prostaglandin biosynthesis by inhibiting cyclooxygenases COX-1 and COX-2. It was first discovered in 1963 and its mechanism of action, along with several other NSAIDs that inhibit COX, was described in 1971. Chemically it is 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetic acid (Hart and Boardman, 1963) an indol-3-acetic acid derivative. It has been found to not only reduce and relieve symptoms of inflammatory arthritis but also in degenerative joint disease (Percy et al, 1964). It is a potent non selective cycloxygenase (COX) inhibitor, which is known to inhibit both COX-1 and COX-2 (Barnett, Chow et al. 1994). Indomethacin has been reported to regulate the production and secretion of matrix metalloproteinases (Ito, Nose et al, 1995; Corcoran, Stetler-Stevenson et al, 1992).

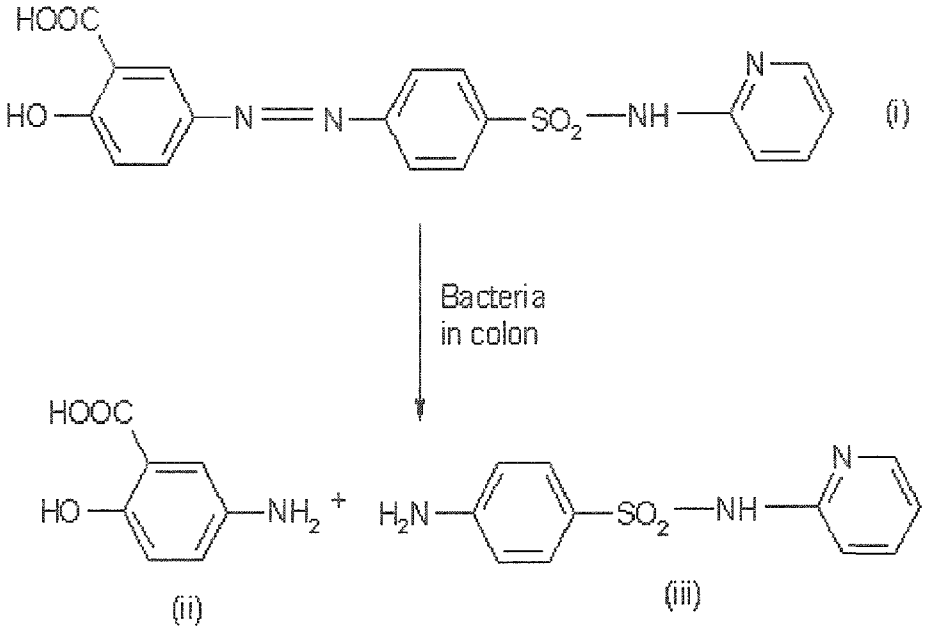
1.2.3.2 Indomethacin Heptyl Ester

Within the indomethacin structure the carboxylate can be derivatized as an ester or amide. These derivatives show enhanced selectivity for the COX-2 isoform (Barnett et al, 1994). A great number of indomethacin derivatives have been developed over the years, one particular derivative, known as indomethacin heptyl ester is known to act as a potent and selective inhibitor of COX-2 and only has a trivial effect on the activity of COX-1 (Kalgutkar et al, 2000).

1.2.4 Disease modifying anti-rheumatic drugs (DMARDs)

Diseases modifying anti-rheumatic drugs (DMARDs) are widely used in the treatment of autoimmune disorders such as rheumatoid arthritis and are designed to slow down the disease progression. They are used early in the treatment of patients with early onset RA. They are also used for a number of other inflammatory diseases such as Crohn's disease and lupus erythematosis (SLE). They are a large group of drugs whose members include sulfasalazine (SSZ), methotrexate (MTX), leflunomide and cyclosporine A. They are used to reduce pain, swelling and stiffness; however the effect is not immediate and often takes a number of weeks to work.

Figure 1.6 The hydrolysis of sulfasalazine. Sulfasalazine (i) is broken down in the small intestine by bacterial azo reductase producing 5-aminosalicyclic acid (ii) and sulfapyridine (iii).



1.2.4.1 Sulfasalazine (SSZ)

Sulfasalazine was first developed in the late 1930's by pioneering Swedish physician, Professor Svartz (figure 1.6) (Svartz, 1942). SSZ is made up of sulphonamide, a sulfa antibiotic formerly used in the treatment of RA known as sulfapyridine, which is bound with a salicylate, an anti-inflammatory drug known as 5-aminosalicylic acid, otherwise known as mesalazine by an azo bond. After ingestion of the drug ~30% of the drug is absorbed into the body unaltered, whilst the remainder travels to the small intestine where azo bacterial reductase splits the azo bond, breaking the sulfasalazine down to its two

constitutive units. It remains unclear exactly which unit of sulfasalazine is responsible for the anti-inflammatory effect seen after treatment with sulfasalazine (Bird, 1995). Sulfasalazine is known to suppress NF-κB activity by the direct inhibition of IκB kinases α and β (Weber et al, 2000).

1.2.5 Anti inflammatory actions of Statins

Hydroxymethylglutaryl – Coenzyme A (HMG-CoA) reductase inhibitors otherwise known as statins are a family of chemically related molecules that can lower lipids. Used extensively in medical practice they have been shown to reduce cardiovascular morbidity and mortality (Palmer et al, 2004). Statins, pravastatin, a hydrophilic drug and mevastatin and simvastatin, both hydrophobic in nature, are able to inhibit hydroxymethylglutaryl – Coenzyme A (HMG-CoA) reductases. These enzymes catalyse the conversion of HMG-CoA to mevalonic acid during cholesterol biosynthesis. Downstream metabolites, including geranylgeranyl pyrophosphate and farnesyl pyrophosphate regulate prenylation within several critical signalling pathways. Clinical and experimental evidence indicates that the beneficial effects of statins may result from anti-inflammatory mechanisms independent of their lipid lowering effects (Palmer et al, 2004).

1.2.5.1 Pravastatin

Pravastatin is hydrophilic in nature and is rapidly absorbed by the body. On average, 34% of the orally administered dose is absorbed by the body. After absorption, 66% of pravastatin undergoes a first-pass extraction through the liver, which is the primary site of its action and the primary site of cholesterol synthesis and clearance of LDL-cholesterol (Kwak et al, 2000).

1.2.5.2 Simvastatin

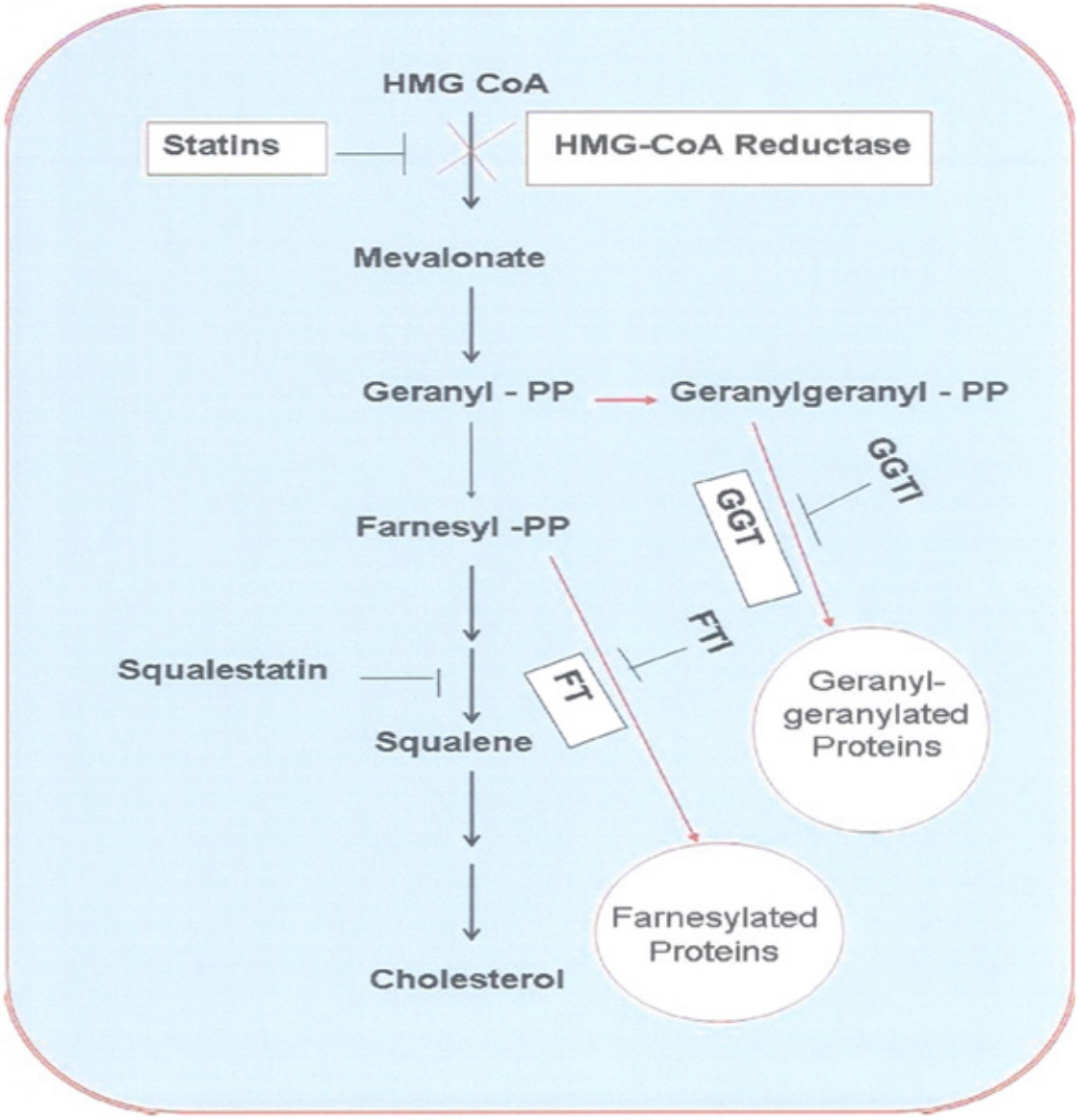
Simvastatin is a hydrophobic member of the statin family. After oral ingestion, simvastatin, which is an inactive lactone, is hydrolyzed in the liver to the corresponding active beta hydroxyacid form which has a potent activity in inhibiting HMG CoA reductase (3 hydroxy – 3 methylglutaryl CoA reductase). This enzyme catalyses the conversion of HMG CoA to mevalonate, an early and rate-limiting step in the biosynthesis of cholesterol. Simvastatin is well absorbed and undergoes extensive hepatic first-pass extraction. The extraction in the liver is dependent on the hepatic blood flow. The liver is

the primary site of action of the active form. The availability of the beta hydroxyacid to the systemic circulation following an oral dose of simvastatin was found to be less than 5 % of the dose (Yada et al, 1999).

1.2.5.3 Mevastatin

Mevastatin is another member of the hydrophobic statin family; it is an antibiotic that acts as a potent inhibitor of HMG-CoA reductase, it suppresses Ras farnesylation and inhibits myoblast fusion (Sugiyama et al, 2000). Mevastatin is not licensed for use in the United Kingdom.

Figure 1.7 Statin-sensitive metabolic pathway.

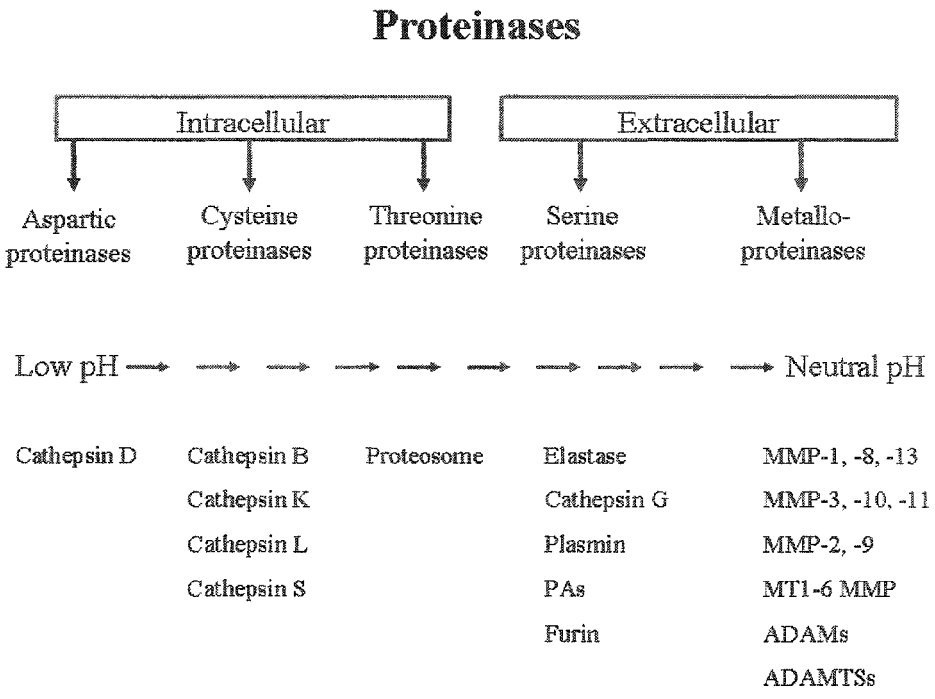


1.3 CARTILAGE DEGRADATION

1.3.1 The homeostasis of normal human cartilage

Normal human cartilage exists in a steady, tightly controlled state of tissue turnover and biochemical homeostasis, which is achieved due to a balance between degradation and synthesis of essential cartilage components. The processes are tightly regulated so as to control highly degradative factors such as proteinases, which are controlled at a number of levels: synthesis, secretion, activation and inhibition (Rowan, 2001). This control is achieved primarily through chondrocytic response to environmental changes such as pH or mechanical loading, or the actions of cytokines, growth factors or small molecules such as nitric oxide.

Figure 1.8 Classes of proteinases



1.3.2 Proteinases involved in matrix turnover

There are five main classes of proteinases, of which three act intracellularly (aspartate, cysteine and threonine) and two which are largely extracellular (metallo and serine) (figure 1.7). Intracellular proteinases are predominantly active at an acidic pH whilst the

extracellular proteinases are active at a neutral pH. Some of the extracellular proteinases are membrane bound and are linked with cytokine processing, receptor shedding and the removal of proteins which are involved in cell-to-cell and cell-to-matrix protein interactions. Other proteinases are not involved directly in the cleavage of matrix proteins but are able to activate proenzymes which work to degrade the matrix (Cawston et al, 2006).

1.3.3 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of over 23 enzymes which are known to facilitate extracellular matrix turnover and breakdown during normal and disease conditions (Kevorkian et al, 2004). All MMPs contain common domains (figure 1.8), with a typical MMP having a propeptide domain consisting of around 80 amino acids, a catalytic metalloproteinase domain with about 170 amino acids, a linker peptide of varying lengths which is also known as the hinge region and a hemopexin (Hpx) domain of about 200 amino acids. However, there are exceptions to this with MMP-7, -23 and -26 which lack both the linker peptide and the hemopexin domain; MMP-23 also has a unique cysteine-rich domain and an immunoglobulin-like domain, which is found after the catalytic domain. The MMPs have other common properties which include a zinc binding motif HEXXHXXGXXH in the catalytic domain and the "cysteine switch" motif PRCGXPD in the peptide domain. When the three histidines in the zinc binding motif coordinate and the cysteine in the propeptide coordinated with the catalytic zinc ion, this Cys-Zn2+ coordination keeps proMMPs inactive (Nagase et al, 2006). MMPs are subdivided into groups such as collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs).

1.3.3.1 Collagenases

The interstitial collagenases MMP-1, -8 and -13, are the enzymes which are uniquely capable of cleaving the three alpha chains of fibrillar collagens such as collagens I – III, and VII (Cawston, 2006). The cleavage of these collagens, in particular collagen type II by these interstitial collagenases suggests that they play a pivotal role in the degradation of the extracellular matrix (Rannou et al, 2006). The degradation of collagen type II is a major, early and irreversible event which generally takes place in the superficial cartilage layer, in the spaces surrounding chondrocytes.

Fibrillar type II collagen is initially broken down by MMP-1 and MMP-13, which are found to be expressed at high levels, however whilst MMP-8 is known to be highly expressed in vitro by chondrocytes stimulated with interleukin-1β, its role in vivo remains unclear (Aigner et al, 2001). MMPs -1 and -13 both differ in their affinity for collagen II and the localisation of their expression distribution within the cartilage matrix, with MMP-1 being expressed mainly in the superficial layer whilst MMP-13 is expressed mainly in deep cartilage (Rannou et al, 2006).

Figure 1.9 Domain structures of matrix metalloproteinases (MMPs)

All metalloproteinases have a catalytic domain, zinc-binding domain (Zn) and propeptide (Pro). Some contain a furin recognition motif (Fu), a fibronectin-like domain (F), a vitronectin-like domain (V), a transmembrane domain (TM) with a cytoplasmic tail (Cyt), a glycosylphosphatidyl inositol (GPI). MMP-23 is unique and possesses a cysteine array (CA) and an immunoglobulin domain (Ig-like). (Clark and Parker, 2003)

Group 1	MMP-7,-26	Minimal domain	Pre Pro Catalytic Zn
Group 2	MMP-1,-3,-8, -10,-12,-13,-18, -19,-20,-22,-27	Simple hemopexin domain	Pre Pro Catalytic Zn Hemopexin
Group 3	MMP-2,-9	Gelatin binding	Pre Pro Catalytic F F F Zn Hemopexin
Group 4	MMP-11,-28	Furin activation, secreted	Pre Pro Fu Catalytic Zn Hemopexin
Group 5	MMP-21	Vitronectin insert	Pre Pro V Fu Catalytic Zn Hemopexin
Group 6	MMP-14, -15,-16,-24	Transmembrane MMP-s	Pre Pro Fu Catalytic Zn Hemopexin TM Cyt
Group 7	MMP-17,-25	GPI anchored MMPs	Pre Pro Fu Catalytic Zn Hemopexin GPI
Group 8	MMP-23	Cys/Pro rich with Ig-like domain	Pre Pro Fu Catalytic Zn CA Ig-like

1.3.3.2 Gelatinases

Gelatinases, MMP-2 and MMP-9 cleave denatured interstitial collagen, as well as collagens IV and V (Rannou et al, 2006) and are important for the breakdown of basement membranes. MMP-2 is thought to be the most widely distributed of all of the MMP family and is produced by a variety of cells which includes chondrocytes. MMP-2 is also able to activate proMMP-13 (Cawston et al, 2006). It is regulated differently from the other MMPs both at a transcriptional and extracellular level and unlike most other MMPs where activation occurs in the extracellular milieu, the activation of MMP-2 occurs on the cell membrane (Jackson et al, 2001) by MT1-MMP in complex with TIMP-2. MMP-9 is known to be expressed by transformed and tumor-derived cells, neutrophils and monocytes (Cawston, 1996). The regulation of MMP-9 is strictly controlled at a number of levels: gene transcription, synthesis, secretion, activation, inhibition and glycosylation. The activation of MMP-9 occurs due to the removal of the propeptide domain by proteolysis. A number of proteases are known to activate MMP-9 and these include MMP-3 and MMP-2 (Ram et al, 2006).

1.3.3.3 Stromelysins

Stromelysins are MMPs with broad substrate specificity; their substrates included proteoglycans, gelatin, fibronectin and collagen type IX (Rannou et al, 2006). MMP-3 and MMP-10 have very similar substrate specificity but have very distinct tissue expression patterns; however both are able to activate latent collagenases (Cawston et al, 2006). MMP-3 is not normally widely expressed, but is readily induced by growth factors and cytokines such as interleukin-1 (MacNaul et al, 1990). Its optimum pH is relatively low and it is thought that the activation of MMP-3 may occur due to long exposure to acidic conditions (Cawston et al, 1996) as well as by serine proteinase plasmin (Milner et al, 2001). MMP-10 like MMP-3 is induced by inflammatory cytokines, and it is thought to play a role in extracellular matrix remodelling. The serine proteinase plasmin may also play a role in the activation of MMP-10 (Barksby et al, 2006) and MMP-3. MMP-11 is found to be expressed in stromal breast adenocarcinoma tissue and in other carcinomas, and has low proteolytic activity for connective tissue components (Cawston, 1996).

1.3.4 Tissue inhibitors of metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinases (TIMPs) work by inhibiting all active MMPs and they do this by tightly binding to the active MMP in a 1:1 ratio. TIMPs are able to control

connective tissue breakdown; if the level of TIMP exceeds that of the active enzymes connective tissue breakdown can be prevented (Cawston et al, 2006). There are currently four TIMPs: TIMP-1 and TIMP-3 expression is known to be inducible, whilst TIMP-2 expression is constitutive. TIMP-4 however, is highly regulated and restricted to neural tissue, foetal testes, and sertoli cells or ovaries, as well as cardiac, breast and skeletal muscle tissues (Young et al, 2002; Lambert et al, 2004). All TIMPs have six disulphide bonds that hold the protein in two domains (Verstappen and Von den Hoff, 2006).

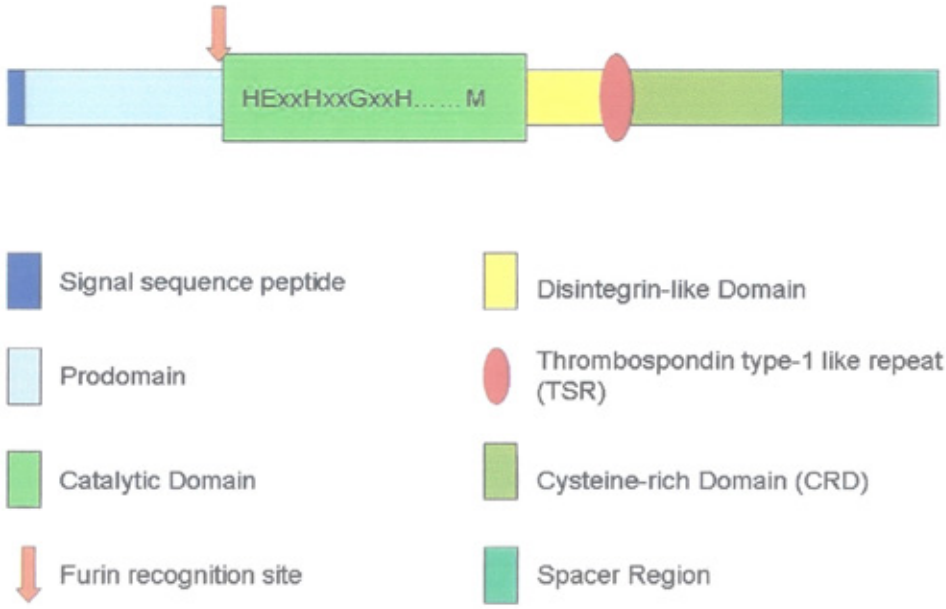
1.3.5 ADAMTS (A Disintegrin And Metalloproteinase with ThromboSpondin repeats)

The ADAMTSs include 19 members and develop as nonintegral membrane proteins which connect with the cell surface and ECM through specific protein domains (Wight, 2005). They are multi-domain proteases which have common structural motifs (figure 1.9).

The N-terminal signal sequence of ADAMTS is followed by a pro-domain, which contains at least one furin cleavage consensus motif. Attached to the pro-domain is the catalytic domain. This domain maintains a high level of similarity throughout the ADAMTSs and contains a zinc-binding sequence of HEXXHXXGXXH. Here the catalytic zinc is coordinated by an arrangement of three histidines residues, which is facilitated by a glycine residue. This in turn permits a tight hairpin loop which enables the third histidine to occupy its correct position. Following the catalytic domain is a region with 25 – 45% identity to snake venom disintegrins, though it contains no cysteine arrangement. It is for this reason that this domain is termed disintegrin-like. A well conserved thrombospondin type 1-like repeat (TSR), which is homologous to the type I repeats of thrombospondin 1 and 2 is found between the disintegrin-like domain and the cysteine-rich domain (CRD). The cysteine-rich domain contains a sequence of 10 cysteine residues, this region is then followed by a cysteine-free 'Spacer' region, which varies in length and contains several conserved hydrophobic residues in the N-terminal portion and an extremely variable C-terminal portion (Jones and Riley, 2005).

Functionally, the ADAMTS proteins fall into a number of groups. The hyalectanases which include ADAMTS-1, -4 and -5, function by cleaving the hyaluronan-binding proteoglycan and the pro-collagen N-propeptidases which include ADAMTS-2, 3 and 14 and cleave amino peptides of procollagens are two of the groups (Jones and Riley, 2005).

Figure 1.10 Domain organisation of ADAMTS



1.3.6 Cartilage Catabolism

In normal cartilage a balance exists between the synthesis and degradation of matrix components and the cartilage is said to be in a 'steady state'. This balance can however easily be changed and even a small change to this steady state can have an effect on the condition of the tissue and this is largely down to the action of pro-inflammatory cytokines within the joint (Rowan, 2001). The study of pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNFα) and their role in the action of inflammatory responses which ultimately leads to a second cytokine cascade and the production of degrading factors such as MMPs has been largely documented (Viviani et al, 2007; Christodoulou et al, 2006) and the loss of the enzyme/inhibitor balance is thought to be a major event in cartilage pathology.

1.3.6.1 Cytokine cascade in joint disease

Interleukin-1 and tumor necrosis factor-alpha are known to play a major part in the destruction of cartilage, however a number of other cytokines and growth factors are also highly expressed and these include IL-6, -8, -10, -11, -13, -17 and -18, and transforming growth factor β (TGFβ). Other cytokines which have also been found, but at much lower

levels, are IL-2, -3, -12 and -15, interferon γ (IFN-γ) and oncostatin-M (OSM) (Rowan, 2001).

During joint diseases such as RA, the synovial membrane undergoes hyperplasia and increased vascularity and is infiltrated by inflammatory cells such as CD4+ T cells. The CD4+ T cells stimulate monocytes, macrophages and synovial fibroblasts causing them to produce numerous inflammatory cytokines including IL-1, TNF-α and OSM and to secrete MMPs through cell surface signalling by means of CD69 and CD11 and through the release of soluble mediators such as interferon-γ, and IL-17 (Isler et al, 1993). The activated CD4+ T cells also stimulate B cells through cell surface contact and through binding of $\alpha_1\beta_2$ integrin. Activated CD4+ T cells express osteoprotegerin ligands that stimulate osteoclastogenesis, these T cells have been shown to cause joint damage in an animal model of RA (Kong et al, 1999). A number of studies have also been carried out using a variety of arthritis animal models to test anti-cytokine therapy and have successfully been able to target not only TNF and IL-1, but also inhibiting IL-15, IL-17, IL-18 and IL-23 (Richards, 2004)

Both TNF-α and IL-1 have major roles to play in rheumatoid pathogenesis and work as stimulators of mesenchymal cells such as chondrocytes and osteoclasts. These cells release MMPs, and produce less TIMPs resulting in cartilage destruction (Choy and Panayi, 2001).

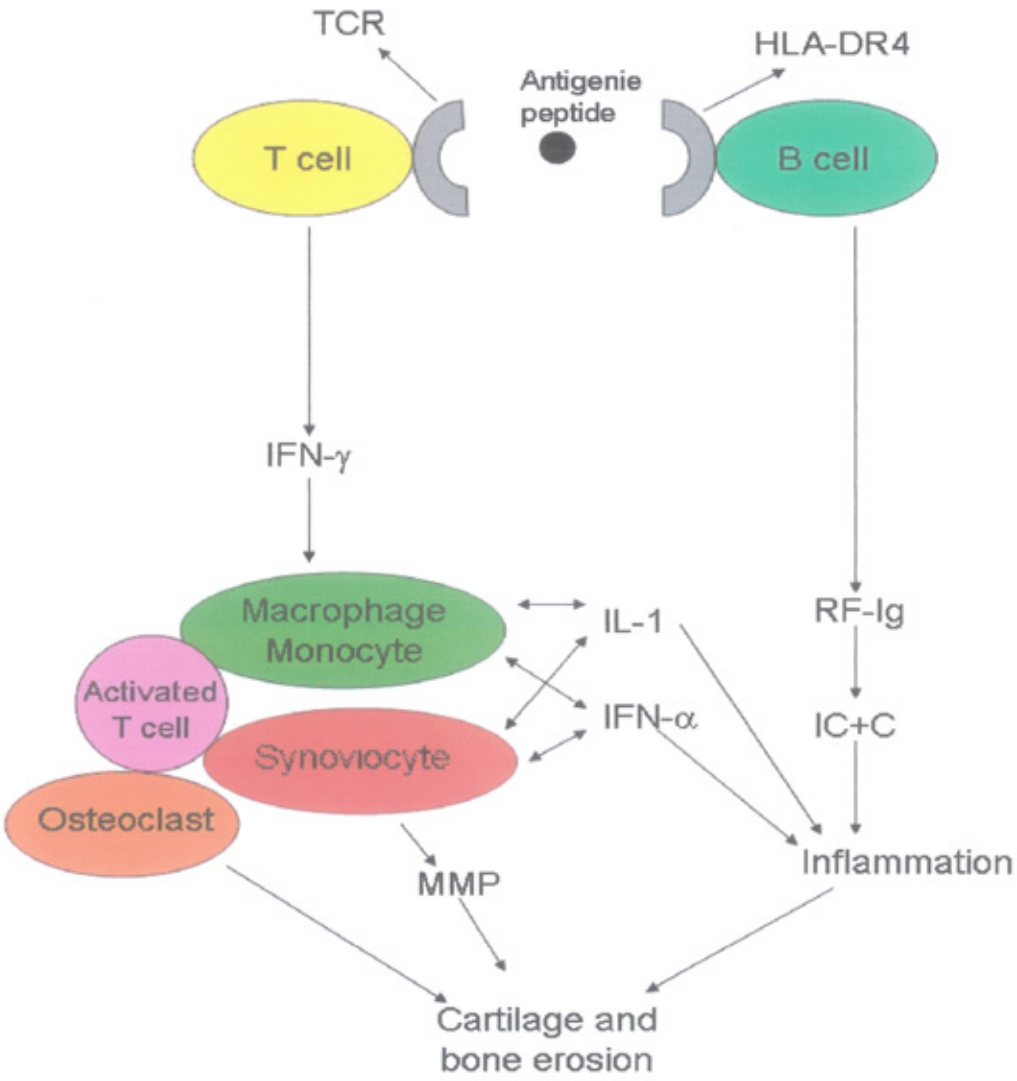
1.3.6.2 Interleukin-1

Interleukin-1 is predominantly, produced by monocytes, macrophages, endothelial cells, B cells and activated T cells (Choy and Panayi, 2001). IL-1 is a 17kd protein and binds to two types of cell-surface receptors (IL1R) type I and type II. Only type I have a cytoplasmic tail and are capable of intracellular signalling (Sims et al, 1993). The type II receptors act as a decoy receptor, by binding circulating interleukin-1 but not delivering any intracellular signals (Colotta et al, 1993). Both IL-1 receptors in their soluble form compete with the receptors on the cell-surface, decreasing IL-1 mediated activation of cells. IL-1 antagonist, a naturally occurring antagonist binds the type I receptor without triggering a signal providing a mechanism for IL-1 activity inhibition (Choy and Panayi, 1993).

Animal arthritis model studies have shown that IL-1 plays a major role in joint destruction, as after injecting IL-1 into rabbit joints, cartilage is found to be highly degraded (Pettipher

et al, 1986). IL-1 concentrations have also been found to be high in the synovial fluid from RA patients, and are thought to cause a high release of MMPs.

Figure 1.11 Cytokines and inflammation in rheumatoid arthritis



TCR = Tcell receptor, IFN-γ = Interferon gamma, IL-1 = interleukin-1, TNF-a = tumor necrosis factor alpha, RF = rheumatoid factor, Ig = immunoglobulin, IC = immune complexes, C = complement, MMP = matrix metalloproteinases

1.3.6.3 Oncostatin M

Oncostatin M (OSM) is a 28kd glycoprotein and a polyfunctional cytokine produced mainly by activated T lymphocytes, monocytes and macrophages (Langdon et al, 1997). OSM is a member of the IL-6 family of cytokines, which are known to control

differentiated cell function and proliferation. Studies have shown that OSM plays a role in inflammatory response and is therefore found in a number of inflammatory diseases such as RA and multiple sclerosis. OSM has been associated with tissue damage as it stimulates the proteolytic pathway and induces the production of inflammatory mediators such as prostaglandin E_2 (PGE_2) and MMPs (Langdon et al, 1997). Interestingly OSM is able to induce a synergistic response with IL-1 and TNF, resulting in the super induction of MMPs by the cells (Richards, 2004). In chondrocytes particularly, the synergistic effect which occurs when OSM is in combination with either IL-1 or TNF appears to occur through the induction of the expression of collagenase, stromelysin-1, and aggrecanases ADAMTS-4 and -5 (Koshy et al, 2002)

1.4 THE NUCLEAR FACTOR-κB PATHWAY

1.4.1 The role of NF-κB in disease

Transcription factor NF-κB is know to regulate a number of genes which play an important role in inter- and intracellular signalling, cellular stress responses, cell growth, survival and apoptosis (Hoffman et al, 2002). NF-κB has been shown to be crucial in the induction of inflammatory responses and resulting in the chronic activation of the immune system. This system of inflammatory activation is known to be involved in a wide variety of diseases such as asthma, inflammatory bowel disease, and rheumatoid arthritis. The cytokine cascades, which occur as a stress response to inflammation lead ultimately to NF-κB activation and thus contribute to these diseases. This has led to NF-kB becoming an obvious target for immunosuppressive therapies (De Bosscher et al, 2003).

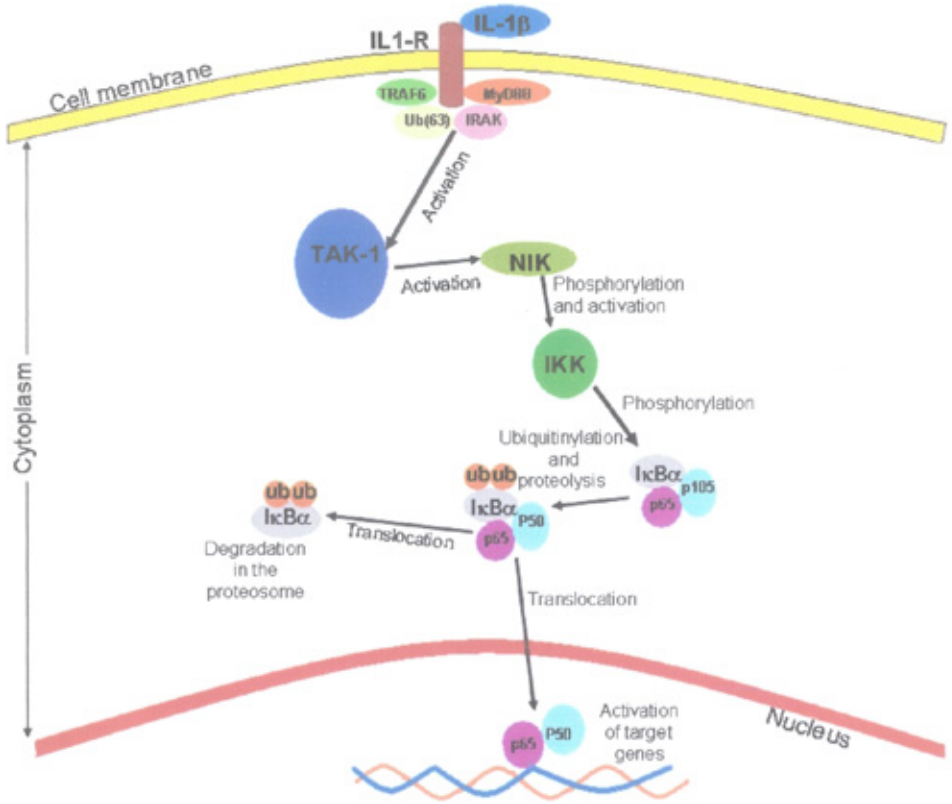
1.4.2 NF-κB signalling by Interleukin-1

NF-κB transcription factors are homo or heterodimers found in the cytoplasm of most resting human cells. The NF-κB family is a group of structurally related and evolutionary conserved proteins which includes NF-κB1 (p50), NF-κB2 (p52), p65 (RelA), RelB and c-Rel (Ghosh and Karin, 2002; Morel and Berebaum, 2004). All members have a conserved stretch of 300 amino acids, known as a Rel homology domain (RHD). The RHD is important for DNA binding, dimerization and association with the IκB inhibitory proteins (Ghosh and Karin, 2002). Although most NF-κB dimers are activators of transcription, the

p50/p50 and p52/p52 homodimers can repress the transcription of their target genes (Zhong et al, 2002).

Figure 1.12 Interleukin-1 NF-kB pathway

Activation of the NF-κB pathway by IL-1. IL-1 receptor (IL-1R), TNF receptor-associated factor 6 (TRAF6), Myeloid differentiation primary response gene 88 (MyD88), IL-1R-associated kinase (IRAK), Ubiquitin 63 (Ub63), TGFβ activating kinase (TAK-1), NF-κB–inducing kinase (NIK), IκB kinase (IKK), κB inhibitor (IκB).



The IκBs are members of a gene family which is made up of seven known mammalian members IκBα, IκBβ, IκBε, IκBγ, Bcl-3 and precursor Rel proteins p100 and p105 (figure 1.11). IκB is characterised by the presence of multiple ankyrin repeats which are protein-protein interaction domains that interact with NF-κB via the RHD to form IκB: NF-κB complexes (Ghosh et al 1998). The structure of IκB: NF-κB complexes reveals the IκB ankyrin repeats, which consists of two closely packed helices followed by a loop and a tight hairpin turn. The repeats stack in a slightly curved cylinder with loops forming

finger-like extensions and the dimerization domains of the NF-κB dimers are able to form an interaction with these loops (Ghosh and Karin, 2002).

Activation of the NF-κB occurs due to the stimulatory effect of cytokine such as TNFα and most notably IL-1. Interleukin-1 binds to its receptor and activates TGFβ activating kinase (TAK-1), which in turn activates NF-κB–inducing kinase (NIK). NIK triggers the phosphorylation and activation of the κB inhibitor (IκB) via the activation of IκB kinase (IKK). Under normal conditions IκB binds to the transcription factor NF-kB, which is composed of subunits p105 and p65. After stimulation IκB phosphorylation takes place which results in the ubiquitinylation of the molecule. IκB then undergoes degradation in the proteosome, whilst p105 undergoes proteolysis to p50, causing the release of the NF-κB complex (p50/p65), allowing its translocation to the nucleus where it is able to activate its target genes. Within arthritis it is this pathway which is necessary for MMP-13 expression and MMP-1 transcription by the chondrocytes (Morel and Berenbaum, 2004).

1.5 MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY (MAPK)

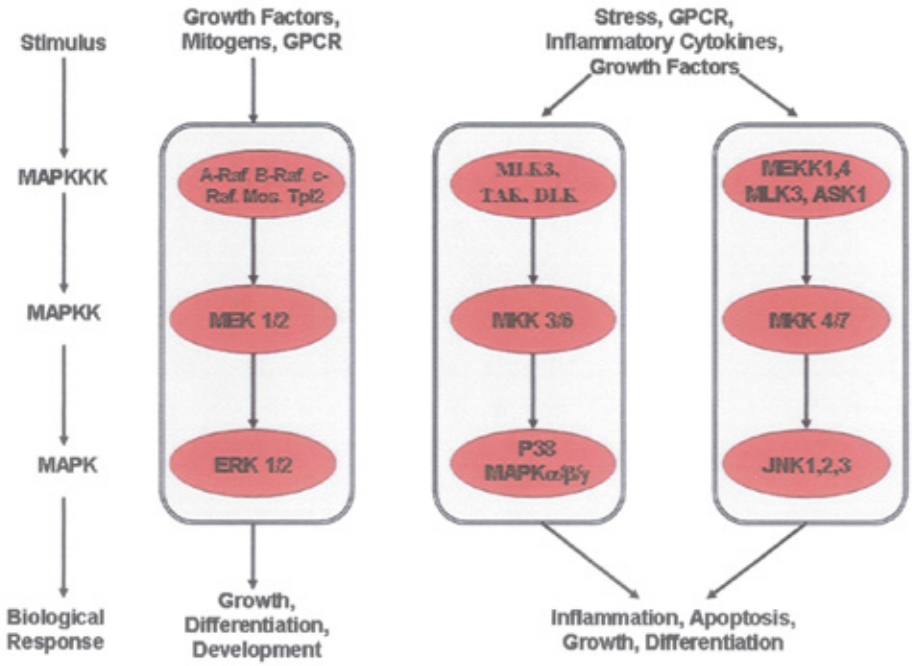
1.5.1 MAPK Pathways

MAPKs are divided into three families, the extracellular signal-regulated kinases (ERK 1/2), c-Jun-N-terminal Kinase (JNK), and p38 (figure 1.12). Mitogens and growth factors primarily activate ERK 1/2, whilst proinflammatory cytokines IL-1 and TNF-α, as well as cell stress-inducing factors such as heat shock, osmotic shock, ultraviolet radiation and oxygen radicals activate the JNKs and p38 (Morel and Berenbaum, 2004). MAPK activation occurs upon phosphorylation of a threonine (Thr) or tyrosine (Tyr) located in a ThrXTyr motif where X is any amino acid (Doza et al, 1995). MAPK activation is catalysed by MAPK kinase (MAPKK or MEK) previously activated by a MAPKK kinase (MAPKKK or MEKK). MAPKKK is activated directly by a member of the small G proteins family such as Ras or via another kinase located upstream in the cascade (Morel and Berenbaum, 2004).

In chondrocytes, IL-1 or TNF-α activates TAK-1 triggering a cascade causing a number of kinases to undergo phosphorylation, and ultimately activates a MAPK complex. Once activated the MAPK complex translocates to the nucleus, where upon it activates and phosphorylates a number of transcription factors such as AP-1 and C/EBP. All three

MAPK complexes can be activated by IL-1 within chondrocytes, with the JNK and p38 being involved predominantly in the activation of MMP-13 expression, whilst ERK 1/2 and p38 mediate MMP-1 expression (Rannou et al, 2006).

Figure 1.13 Mitogen-Activates Protein Kinase signalling cascades



1.5.2 The role of the MAPK pathway in disease

The mitogen-activated protein kinases (MAPKs) which include c-Jun-N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase are activated by a wide range of cellular stresses as well as in response to inflammatory cytokines. Normal cellular proliferation, differentiation and programmed cell death has led to significant recent advances in our understanding of the role of MAPK signalling in inflammatory disorders such as arthritis and cardiovascular disease, cancer, and pulmonary and neurogenerative diseases. Several compounds which are thought to be specific inhibitors of MAPK are already being tested in human subjects to assess their oral bioavailability, pharmacokinetics and toxicity (Malemud, 2007).

In arthritis, MAPK has been shown to be involved in both osteoarthritis and rheumatoid arthritis. In osteoarthritis and rheumatoid arthritis, which are both characterised by degeneration of articular cartilage, MAPK is involved in the regulation of genes, such as

proteinases, cyclooxygenase, and cytokines. Pharmacological inhibitors of the mitogen-activated protein kinases are currently being investigated in animal models of osteoarthritis and in vitro, with the intention of using compounds that block this signalling pathway as a therapy for osteoarthritis (Saklatvala, 2007). In rheumatoid arthritis the MAP kinases are also expressed and activated in the synovium. Preclinical studies using MAP kinase inhibitors have shown that they are effective in animal models, supporting their potential use in human disease for the treatment of rheumatic and other immune-mediated diseases (Sweeney and Firestein, 2006).

1.5.3 c-Jun N-terminal Kinase (JNK)

JNK has been implicated in a wide range of physiological processes, which include cellular responses to stress, however it has also been shown to play a role in embryonic development, cell survival, apoptosis and proliferation (Dong et al, 2001; Davis et al, 2000).

JNK works by phosphorylating a number of substrates which include c-Jun (serines 63 and 73), JunB, JunD, ATF-2 and NFAT, and the activation of JNK is mediated by the dual phosphorylation of tyrosine and threonine by MAPKKs MKK4 and MKK7. Substrate phosphorylation by JNK is thought to increase their transcriptional activity, however recent studies suggest that the overall regulation may be far more complex than a simple up regulation of responses. It has also been shown that inhibition of MKK4 does not affect the ability of JNK to increase AP-1 activity (Yang et al, 1997). The phosphorylation of c-Jun at ser63 and 73 has also been shown to inhibit degradation and increase overall transcriptional activity (Fuchs et al, 1996). The activation of the JNK pathway due to stress may suggest that JNK aids an inflammatory response and it has also been shown to induce apoptosis (Seimiya et al, 1997)

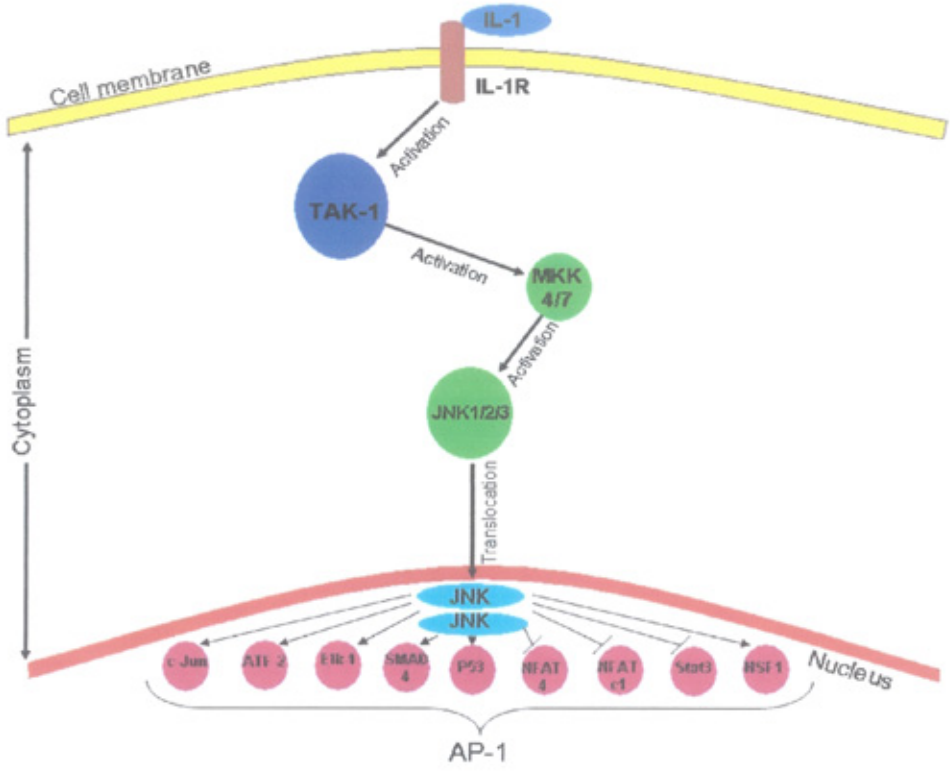
1.5.4 AP-1 Pathway

The transcription factor AP-1 is encoded by proto-oncogenes and regulate various aspects of cell proliferation and differentiation. They are composed of combinations of homo- and heterodimers between members of the fos (c-fos, fos-B), jun (c-jun, v-jun, jun-B and jun-D) and ATF (ATF-2, ATF-3) families (De Bosscher et al, 2003). The members vary in amount across tissues, which influence the nature of their interactions with other regulators of transcription (Rannou et al, 2006). The regulation of AP-1 activity is complex and a number of factors play a role in its activity. These factors include, changes in jun and fos

gene transcription and mRNA turnover, the effects of jun and fos on protein turnover; the posttranslational modifications of jun and fos proteins that modulate their transactivation potential and transcription factors which cause synergy or interference (De Bosscher et al, 2003). AP-1 activity is modulated by interactions from other transcriptional regulators which are controlled by upstream kinases that link AP-1 to various transduction pathways. The expression of ATF, particularly ATF-2 is thought to be constitutive and its activity is regulated by protein phosphorylation by JNK and p38 (Triesman, 1996). Stimuli such as growth factors, mitogens, cell-matrix interactions and cytokines have been shown to induce AP-1 activity, inducing the activation of MAPK cascades which enhance AP-1 activity by phosphorylating distinct substrates (Wagner, 2002; De Bosscher et al 2003)

Figure 1.14 AP-1 signal pathway involving control of MMP expression

IL-1 receptor (IL-1R), TGFβ activating kinase (TAK-1)MAPK kinase kinase 4 (MKK4), c-Jun N-terminal kinase (JNK), activator protein 1 (AP-1), C-Jun N-terminal kinases (c-Jun), Activating transcription factor-2 (ATF2), protein 53 (p53), Nuclear factor of activated T-cells (NFAT), signal transducer and activator of transcription (STAT), Heat shock factor (HSF).



The components of AP-1 are known to be more highly expressed in RA compared to OA and a clear role for AP-1 has been demonstrated during the transcription of MMP-1, -3 and -13, where the structural similarity in the proximal region of human MMP-1 and MMP-13 genes contain an AP-1 site, therefore allows AP-1 binding. A number of studies have been carried out investigating the implications of AP-1 in arthritis, where animal studies have shown that by blocking AP-1 in vivo joint destruction can be inhibited, whilst disease onset has been shown after the activation of AP-1 (Shiozawa et al, 1997; Han et al, 1998). Another study has shown that ATF-2 and c-fos appear to be crucial for MMP-1 activity with only ATF-2 inhibiting c-jun transcription and suggesting that ATF-2-containing dimers are likely to be important to the overall AP-1 response (Steinmuller et al, 2001).

1.7 SUMMARY AND AIMS OF THIS STUDY

The breakdown of cartilage matrix is a hallmark of rheumatic disease. The rapid but reversible loss of proteoglycan and the slow but permanent loss of collagen are vitally important steps in cartilage tissue breakdown. Members of the matrix metalloproteinase family such as the collagenases are known to be key enzymes in the destruction of cartilage collagen and have been found to be highly expressed in the synovial fluids of arthritis patients.

Very little is known about the mechanism by which drugs such as DMARDs and NSAIDs retard the damage to joint cartilage. The aim of this study is to investigate the effect of these drugs on the loss of proteoglycan and collagen during the process of cartilage degradation as well as to investigate the mechanism by which the drugs work.

The synergistic destruction of cartilage by IL-1 and OSM was reported by Cawston et al (1995) using the bovine nasal cartilage model to measure the loss of cartilage collagen and proteoglycan from the cartilage after treatment with these inflammatory cytokines. In this study the bovine nasal cartilage model is used to screen specific drugs and investigate if they are able to block the release of collagen and/or proteoglycan from the cartilage. Once a possible inhibitory effect is found, human articular chondrocytes, digested from human cartilage, were stimulated with IL-1 and OSM to induce a destructive effect and drugs added in order to measure the changes in protein and gene expression levels of MMPs and the effects on signalling pathways due to the inhibitory effect of the drug. This study tests the hypothesis that drugs used in the treatment of the pain and inflammation found in arthritic disease also provide chondroprotective effects by blocking key mechanisms of

cartilage breakdown and so preventing the destruction of articular cartilage by matrix degrading enzymes.

The aims of this work were to:

- Test the hypothesis that drugs used to treat inflammation and pain in arthritis may also have chondroprotective properties.
- Study different classes of drugs on their ability to prevent the release of proteoglycan and collagen during the destruction of cartilage.
- Determine which key enzymes, known to be involved in arthritis, are blocked by specific compounds.
- Determine the specific mechanism of action of one compound in preventing joint destruction.

CHAPTER 2: General Methods and Materials

2.1 MATERIALS

2.1.1 Biochemical assay reagents

Bovine serum albumin (BSA), chondroitin sulphate A (from bovine trachea), collagenase (type I from *Clostridium histolyticum)*, L-cysteine hydrochloride, *p*-dimethylaminobenzaldehyde (DAB), dimethylmethylene blue (DMB), hydroxyl-L-proline, o-phenylenediamine (OPD) and phosphate-citrate capsules were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Chloramine T was purchased from BDH (Poole, UK). Maxisorp Nunc-immuno 96-well plates were obtained from Life Technologies Ltd. (Paisley, UK). Flat-bottomed 96-well plates were obtained from Bibby Sterilin (Staffordshire, UK). LP3 tubes were obtained from Life Sciences International Ltd. (Basingstoke, UK). V-bottomed 96-well plates (M25) were obtained from Dynatech Laboratories (Billington, UK). Flexible 96-well sample plates and Optiphase "Supermix" scintillation fluid were obtained from Wallac (Milton Keynes, UK). 2 ml o-ring screw-cap tubes were obtained from Sarstedt (Leicester, UK). In-house antibodies were used for ELISAs.

2.1.2 Cell and tissue culture reagents

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), L-glutamine and penicillin-streptomycin were obtained from Life Technologies Ltd. (Paisley, UK). 24-well, 12-well and 6-well tissue culture plates were obtained from Corning/Costar UK Ltd. (High Wycombe, UK). Cell scrapers were obtained from Greiner Labortechnik (Gloucester, UK). Sterile 100 mm square Petri dishes and universals (20 and 5 ml) were obtained from Bibby Sterilin Ltd (Staffordshire, UK). Collagenase (type I from *Clostridium histolyticum)*, hyaluronidase (type I-S from bovine testes), trypsin (type III, from bovine pancreas), Dulbecco's phosphate buffered saline (DPBS), BSA, gentamicin solution and nystatin suspension were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). Syringe filters (0.2 μm) were from Pall Gelman Sciences (Northampton, UK). Sterile disposable scalpel blades were from Swann-Morton (Sheffield, UK).

2.1.2.1 Cytokines

Recombinant human IL-1 α was a generous gift from Dr Keith Ray (GlaxoSmithKline, Stevenage, UK). Recombinant human OSM was kindly donated by Prof. John Heath (Department of Biochemistry, University of Birmingham, UK). IL-1 α at 1 μg/ml in DMEM (with 0.1% BSA) was stored at -20°C. OSM at 10 μg/ml in DPBS (containing 0.1% BSA) was stored at -80°C. Immediately prior to use IL-1 α and OSM were diluted in culture medium and sterile filtered through a 0.2 μM filter.

2.1.3 Molecular Biology reagents

RNeasy Mini kit was obtained from Qiagen (Crawley, UK). Random hexamers ($pd(N)_6$), 5X First-Strand Buffer, SuperScript™ II RT, DTT, dNTP mix and RNaseOUT™ Recombinant Ribonuclease Inhibitor were obtained from Invitrogen™ Life Technologies (Paisley, UK). Water was obtained from Sigma-Aldrich Company Ltd. (Poole, UK).
All reagents required for RNA work were molecular biology grade. All RNA work was performed under RNase-free conditions using RNase-free reagents and materials.

2.1.3.1 Complementary DNA probes

cDNA probes for house keeping gene 18S and TaqMan Universal PCR mix 2X were obtained from Applied Biosystems (Warrington, UK).

2.1.3.2 TaqMan Low Density Arrays

TaqMan Low Density Arrays were custom made for MMPs, ADAMTS and TIMPS and obtained from Applied Biosystems (Warrington, UK)

2.1.4 Protein electrophoresis and Western Blotting reagents

Ammonium peroxodisulphate (APS) (2 g sachets) and gelatin were obtained from BDH (Poole, UK). β-mercaptoethanol, N,N,N'N'–tetramethylenediamine (TEMED), 1,10-phenanthroline and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich Company Ltd. (Poole, UK). 40% (w/v) acrylamide/bis-acrylamide (37.5:1) solution was obtained from Anachem (Luton, UK). SDS-PAGE molecular weight standards (low range) and precision protein standards were obtained from Bio-Rad Laboratories Ltd. (Hemel Hempstead, UK). See Blue Plus 2 pre-stained protein standards were obtained from Invitrogen (Netherlands). Enhanced chemiluminescence (ECL)

Western blotting detection reagents and hyper film were obtained from Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK). Streptavidin-HRP and HRP conjugated antibodies were obtained from DAKO Ltd. (Cambridgeshire, UK).

2.1.5 Other reagents

All other chemicals and biochemicals, unless otherwise stated, were commercially available analytical grade reagents obtained from Sigma-Aldrich Company Ltd. (Poole, UK), Fisher Scientific (Loughborough, UK), Life Technologies (Paisley, UK) or BDH (Poole, UK).

2.1.6 Ethics

All human tissue used was received form the orthopaedic surgery unit at Newcastle Freeman Hospital with full ethical approval.

2.2 CARTILAGE EXPLANT CULTURE

Bovine nasal cartilage explant cultures were stimulated to resorb with cytokines IL-1α and OSM as a model of cartilage degradation. Serum was excluded from cartilage explants since it can increase cartilage metabolism in the absence of exogenous cytokine(s) (Sah et al. 1994). The aim was to create a model of cartilage breakdown; therefore, the presence of serum is avoided as it contains chondroprotective agents such as insulin-like growth factor-1 (IGF-1) (Luyten et al. 1988; Tyler 1989). The absence of serum has been shown not to affect the viability of the tissue and previous studies have shown that cartilage in serum-free culture for 8-9 days can respond to serum and other growth factors (Hascall et al. 1983).

2.2.1 Bovine nasal cartilage degradation assay

Solutions

Culture medium

DMEM medium containing 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 µg/ml nystatin.

Dulbecco's phosphate buffered saline (DPBS)

DPBS containing 20 µg/ml nystatin.

Phosphate buffer

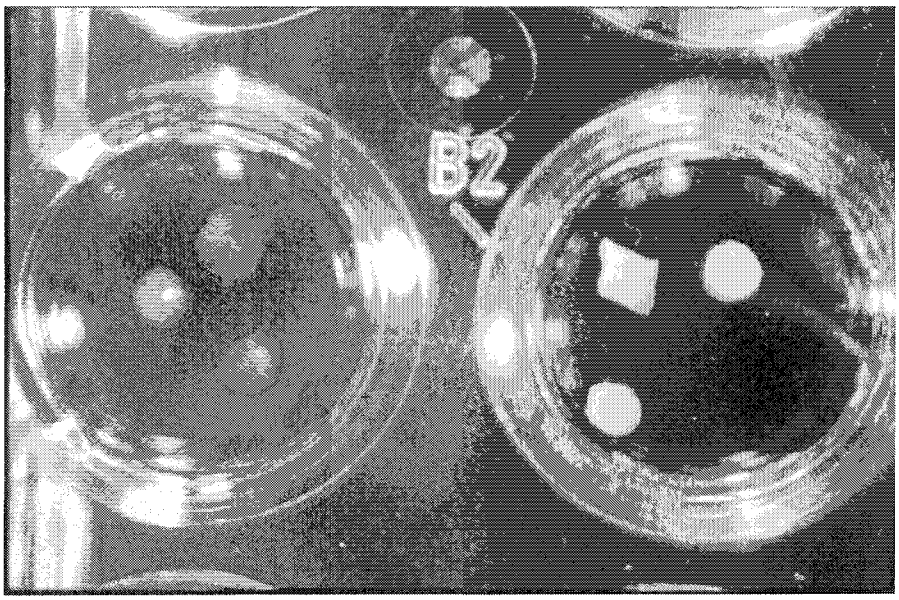
137 ml of 0.1 M NaH_2PO_4 and 63 ml 0.1 M $NaHPO_4$, pH 6.5

Method

Bovine nasal septum was dissected from a bovine nose and the connective tissue sheath was removed from the cartilage. Cartilage was cut into 2 mm thick strips and 2 mm diameter discs were punched using a sterilised hole punch. Care was taken to avoid cartilage with obvious vasculature. Cartilage was washed twice with DPBS. Three discs per well were added to 24-well tissue culture plate, in 1 ml of culture medium. The plates were then incubated overnight at 37°C in 5% CO_2/humidified air to allow explants to equilibrate (figure 2.1).

Medium was removed from each well and replenished with 600 µl fresh culture medium containing the appropriate cytokine(s) and test reagent(s). For each condition 4 wells were used. Plates were incubated at 37°C for 7 days. Supernatants were collected and cartilage discs replenished with identical test reagents to day 0 (unless otherwise stated). The experiment was continued for a further 7 days, and at day 14, supernatants were removed and stored at -20°C in the presence of 0.02% (w/v) sodium azide (NaN_3) until assayed. At day 14, the remaining cartilage explants were placed in capped LP3 tubes and digested overnight at 65°C with papain (4.5 mg/ml), cysteine-HCl (5 mM) and EDTA (5 mM) in 550 µl phosphate buffer. After digestion 450 µl of phosphate buffer and 0.02% NaN_3 was added to each well and then stored at 4°C.

Figure 2.1 Bovine cartilage discs in a nasal cartilage assay



2.2.2 Sulphated glycosaminoglycan assay

Principle: 1,9-dimethylmethylene blue (DMB) is a strongly metachromatic dye. It forms a complex with sulphated GAG which absorbs at A_{530}. Sulphated GAG present in samples was assayed using DMB, based on the method of Farndale et al. (1986).

Solutions

DMB

16 µg/ml 1,9-dimethylmethylene blue, 40.5 mM glycine, 40.5 mM NaCl and 9.5 mM HCl, pH3.0. A_{525} = 0.31. Protect from light and stored at room temperature.

Method

A range of GAG standards (bovine chondroitin sulphate) from 0-40 µg/ml was prepared in phosphate buffer. Supernatants and digest samples diluted in phosphate buffer or standard (40 µl) were plated in duplicate into a 96-well plate. 250 µl of DMB was added and the absorbance read immediately at 530 nm (XFluor4 Sunrise Tecan Microplate reader). The sulphated GAG content of the samples was calculated from the standard curve. The amount of GAG released into the medium was calculated as a percentage of the total. The calculation is as follows:

%GAG release = [GAG in supernatants (µg/ml)]/[total GAG(µg/ml)] x100

Where total GAG is the amount of GAG in each sample (supernatants and digests). The % release of GAG was considered to be representative of the % release of proteoglycan.

2.2.3 Hydroxyproline assay

Principle: The amino acid sequence glycine-proline-hydroxyproline occurs frequently in collagen and hydroxyproline is found in very few other proteins. Therefore, hydroxyproline was assayed as a measure of collagen. Protein was hydrolysed to its constituent amino acids. The assay is based on the oxidation of hydroxyproline by chloramine T to a compound related to pyrrole, and the subsequent condensation of this intermediate with DAB to produce a red colour, which is measured at A_{560}. Hydroxyproline present in samples was assayed using a microtitre modification of the assay described by Bergen and Loxley (1963).

Solutions

Acetate-citrate buffer

420 mM sodium acetate, 130 mM tri-sodium citrate, 26 mM citric acid and 38.5% (v/v) propan-2-ol, pH 6.

4.5 M DAB

20 g diaminobenzidine tetrahydrochloride in 30 ml 70% (v/v) perchloric acid, stored at 4°C.

65.5 mM Chloramine T

0.14 g chloramine T in 2 ml dH_2O.

Method

Supernants or cartilage digests (200 µl) were mixed with 200 µl of 12 M HCl in 2 ml o-ring screw-cap tubes. Samples were then hydrolysed in a hot block for 20 hr at 105°C. The hydrolysates were dried in a centrifugal evaporator using an acid resistant integrated Savant Speed Vac (Life Sciences international, Basingstoke, UK) for 2hrs. Once dry, residue was suspended in 200 µl dH_2O and stored at room temperature until assayed. A 1 mg/ml stock of hydroxyproline was diluted in dH_2O to give a series of standards (0-30 µg/ml). On the day of use, DAB (4.5 M) was diluted 1:3 in propan-2-ol and chloramine T (65.5 mM) diluted 1:4 in acetate-citrate buffer. 40 µl of sample or standard (neat or diluted in dH_2O) was added in duplicate to a 96-well plate. 25 µl of 65.5 mM chloramine T was then added. After 4 min, 150 µl of 1.5 M DAB was added. The plate was then sealed with a plastic plate sealer and incubated for 35 min at 65°C. The plate was then allowed to cool and the absorbance read at 560 nm (XFluor4 Sunrise Tecan Microplate reader). The hydroxyproline content of the samples was calculated from the standard curve. The release of hydroxyproline from cartilage was then calculated using the following equation:

% hydroxyproline release = [hydroxyproline in supernatants (µg/ml)]/[total hydroxyproline (µg/ml)] x100

Where total hydroxyproline is the amount of hydroxyproline supernatants and cartilage digests. The % release of hydroxyproline was considered to be representative of the % release of collagen.

2.3 DIFFUSE FIBRIL ASSAY

2.3.1 Collagenase assay

Principle: Collagenolytic activity present in supernatants from bovine nasal and human articular cartilage explant cultures and cell culture experiments was determined using a 96-well plate modification (Koshy et al. 1999) of the diffuse fibril assay (Cawston and Barrett

1979). Collagen at neutral pH and at temperatures above 25°C forms into fibrils where the individual collagen molecules associate to form a viscous gel. In this assay, collagen which has been acetylated with [^{3}H]-acetic anhydride is allowed to form fibrils. At the end of the assay, undigested collagen fibrils are spun down in the centrifuge and the amount of [^{3}H] in the supernatant gives an estimate of the amount of collagen digested. As well as measuring active collagenase activity, a measure of total collagenase activity can be obtained by adding APMA (0.67 mM) to the assay, which activates latent proMMPs.

Controls:

Total lysis of collagen was obtained by the addition of bacterial collagenase.

Negative control (cacodylate buffer) was used to determine the background counts.

Trypsin control was used to determine maximum susceptibility of the labelled non-helical telopeptides to non-specific proteolytic cleavage and to identify if Denaturation of the triple helix had occurred before the assay. Trypsin activity of between 10-20% lysis of total collagen was acceptable. The linear range of the assay was between 10-80% lysis of total collagen.

Solutions

Tris assay buffer

100 mM TrisHCl, pH7.6, 15 mM $CaCl_2$ and 0.02% (w/v) NaN_3.

APMA

10 mM stock solution APMA: 35.2 mg APMA was dissolved in 200 µl DMSO and made up to 10 ml with 100 mM Tris pH8.5. This was stored at 4°C in a foil wrapped container for up to 3 months.

Tris-APMA assay buffer

At the time of assay, APMA was diluted to 2 mM Tris assay buffer.

Cacodylate buffer (CCA)

25mM sodium cacodylate buffer, pH 7.6, 0.05% (w/v) Brij-35 and 0.02% (w/v) NaN3.

[^{3}H] –acetylated collagen

Acid-soluble type I collagen was extracted and purified from calf skin using the method previously described Cawston and Barratt (1979). The purified collagen was freeze-dried and stored at -20°C. When required the collagen was thawed and redissolved in 0.2 M acetic acid at 4°C. Collagen is radiolabelled with [^{3}H]-acetic anhydride (925 MBq; Amersham Pharmacia Biotech Ltd. (Buckinghamshire, UK)) to a high specificity as described in Cawston et al. (2001). For the assay, a 1 mg/ml solution (50 mM TrisHCl pH

7.6, containing 200 mM NaCl and 0.02% (w/v) NaN_3) with a specific activity of approximately 1 x 10^5 dpm/mg was used.

Trypsin (T)

100 µg/ml trypsin in 1mM HCl. Stored -20°C.

Bacterial collagenase (BC)

100 mg/ml bacterial collagenase in cacodylate buffer. Stored at -20°C

Methods

To a V-bottomed 96-well plate, 50 µl of Tris assay buffer ± APMA was added to each well followed by 10 µl of sample in duplicate (neat or diluted in CCA) + 40 µl cacodylate buffer was then added. The three sets of controls (in duplicate) included in the assay 1) cacodylate buffer (50 µl); 2) 10 µl trypsin + 40 µl cacodylate buffer; 3) bacterial collagenase (50 µl). This was followed by the addition of 50 µl of [^{3}H]-acetylated collagen.

The 96-well plate was incubated at 37°C (16-20hr) then centrifuged at 1056g, 4°C for 30 min in Sorvall RC5C Plus centrifuge. Supernatant (50 µl) was removed and placed in a flexible 96-well plate with 200 µl of Optiphase "Supermix" scintillation fluid. Counts were read in a 1450 Micro-beta Trilux liquid scintillation and luminescence counter (Wallac). Collagenase activity was measured in unit/ml, where one unit can degrade 1 µg of collagen per min at 37°C.

Equation for calculating collagenase activity (unit/ml):

= (50/total lysis) x (1000/sample volume (µl)) x (1/(time (min)) x (sample – blank)

2.4 ENZYME-LINKED IMMUNOSORBANT ASSAYS (ELISAs)

Principle: Monoclonal antibody was immobilised on a Maxisorb Nunc-immuno 96-well plate. Antigen (sample) was added which binds to the antibody. A second antibody conjugated with biotin was then added which recognises the antigen also. Streptavidin-HRP was then added. Streptavidin binds to biotin. OPD was then added which is oxidised by HRP forming a coloured product.

2.4.1 MMP-1 ELISA

Principle: Human MMP-1 was measured using a double-antibody sandwich ELISA (Clark et al. 1992) which allows the detection of free enzyme (pro- and active forms), as well as complexes containing MMP-1.

Solutions

Phosphate buffered saline (PBS) pH7.4

8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 0.02% (w/v) NaN_3.

Wash buffer

8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 150 mM NaCl, 2.7 mM KCl, 0.25 mM thimerosol and 0.1% (v/v) Tween 20, pH 7.0.

Blocking buffer

1% (w/v) BSA in PBS

Protein diluent

0.05% BSA in wash buffer.

Monoclonal antibody to human MMP-1 (RRU-CL1)

2 µg/ml in protein diluent.

Anti-MMP-1 polyclonal antibody (anti-CL1)

0.25 µg/ml in protein diluent.

Goat anti-rabbit HRP

Diluted 1:1000 in protein diluent.

OPD substrate solution

1 tablet (15 mg) of OPD dissolved in 12ml of 0.5 M phosphate-citrate buffer, pH5, and containing 0.03% performate (made by dissolving 1 phosphate-citrate capsule per 100 ml of dH_2O).

Methods

Maxisorb 96-well plates were coated overnight with RRU-CL1 at 4°C (100 µl/well). The plates were washed once with wash buffer and blocked with blocking buffer (200µl/well) overnight at 4°C. The blocked plates were then stored at 4 °C for up to one month. When required, plates were washed three times in wash buffer. Standards (0-50 ng/ml) were prepared by diluting human MMP-1 (0.25 µg/ml) in protein diluent. Samples (either neat or diluted in protein diluent) and standards (100 µl/well) were added to the plates in duplicate and incubated for 2hr at room temperature or overnight at 4°C. The plates were washed three times with wash buffer and incubated for 2hr at room temperature with anti-

CL1 (100 µl/well). The plates were washed three times with wash buffer and incubated for 1 hr at room temperature with goat anti-rabbit HRP (100 µl/well). After washing, OPD substrate solution 100µl/well was added and the plates incubated for approximately 5 -10 min at room temperature. The reaction was stopped by the addition of 3 M H_2SO_4 (50 µl/well) and the absorbance read at 490 nm (XFluor4 Sunrise Tecan Microplate reader). The concentration of MMP-1 in the sample was calculated against the standard curve.

2.4.2 MMP-13 ELISA

MMP-13 was measured using a double-antibody sandwich ELISA developed to assay proMMP-13.

Solutions

Monoclonal antibody to mouse anti-MMP-13

1.25 µg/ml in protein diluent.

Biotinylated anti-MMP-13 monoclonal rabbit antibody

0.13 µg/ml in protein diluent.

Streptavidin-HRP

Diluted 1:1000 in protein diluent.

OPD substrate solution

1 tablet (15 mg) of OPD dissolved in 12ml of 0.5 M phosphate-citrate buffer, pH5, containing 0.03% perforate (made by dissolving 1 phosphate-citrate capsule per 100 ml of dH_2O).

Methods

Maxisorb 96-well plates were coated overnight with anti-MMP-13 at 4°C (100 µl/well). The plates were washed once with wash buffer and blocked with blocking buffer (200µl/well) overnight at 4°C. The blocked plates were then stored at 4 °C for up to one month. When required, plates were washed three times in wash buffer. Standards (0-20 ng/ml) were prepared by diluting human proMMP-13 (2 µg/ml) in protein diluent. Samples (either neat or diluted in protein diluent) and standards (100 µl/well) were added to the plates in duplicate and incubated for 2hr at room temperature or overnight at 4°C. The plates were washed three times with wash buffer and incubated for 2hr at room temperature with biotinylated anti-MMP-13 (100 µl/well). The plates were washed three times with wash buffer and incubated for 30 mins at room temperature with Streptavidin-HRP (100 µl/well). After washing, OPD substrate solution 100µl/well was added and the

plates incubated for approximately 5 -10 min at room temperature. The reaction was stopped by the addition of 3 M H_2SO_4 (50 µl/well) and the absorbance read at 490 nm (XFluor4 Sunrise Tecan Microplate reader). The concentration of MMP-13 in the sample was calculated against the standard curve.

2.4.3 TIMP-1 ELISA

TIMP-1 was measure using a double-antibody sandwich ELISA developed to assay Total TIMP-1.

Solutions

Monoclonal antibody to RRU-T5

5µg/ml in protein diluent.

Biotinylated RRU-T1 monoclonal antibody

0.25 µg/ml in protein diluent.

Streptavidin-HRP

Diluted 1:1000 in protein diluent.

OPD substrate solution

1 tablet (15 mg) of OPD dissolved in 12ml of 0.5 M phosphate-citrate buffer, pH5, containing 0.03% perforate (made by dissolving 1 phosphate-citrate capsule per 100 ml of dH_2O).

Methods

Maxisorb 96-well plates were coated overnight with RRU-T5 at 4°C (100 µl/well). The plates were washed once with wash buffer and blocked with blocking buffer (200µl/well) overnight at 4°C. The blocked plates were then stored at 4 °C for up to one month. When required, plates were washed three times in wash buffer. Standards (0-50 ng/ml) were prepared by diluting human rTIMP-1 (5 µg/ml) in protein diluent. Samples (either neat or diluted in protein diluent) and standards (100 µl/well) were added to the plates in duplicate and incubated for 2hr at room temperature or overnight at 4°C. The plates were washed three times with wash buffer and incubated for 2hr at room temperature with biotinylated RRU-T1 (100 µl/well). The plates were washed three times with wash buffer and incubated for 30 mins at room temperature with Streptavidin-HRP (100 µl/well). After washing, OPD substrate solution 100µl/well was added and the plates incubated for approximately 5 -10 min at room temperature. The reaction was stopped by the addition of 3 M H_2SO_4 (50 µl/well) and the absorbance read at 490 nm (XFluor4 Sunrise Tecan

Microplate reader). The concentration of TIMP-1 in the sample was calculated against the standard curve.

2.5 CELL EXTRACTION AND CULTURE

Principle: Human articular cartilage was used as a source of primary chondrocytes. A three-step enzymatic digest of cartilage to remove the extracellular matrix allowing the chondrocytes to be isolated.

2.5.1 Human articular chondrocyte isolation

Solutions

Culture medium

DMEM medium containing 25 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20 µg/ml nystatin and 10% (v/v) Foetal bovine serum.

Dulbecco's phosphate buffered saline (DPBS)

DPBS containing 20 µg/ml nystatin

Hyaluronidase

1 mg/ml in DPBS

Trypsin

2.5 mg/ml in DPBS

Collagenase

3 mg/ml in culture medium.

Method

Cartilage was removed from fresh joint replacements and washed in DPBS. The amount of cartilage was determined and placed into a pre-weighed falcon tube. Cartilage was incubated at 37°C with hyaluronidase (20 ml/5 g cartilage) for 15 min on an orbital shaker at 110rpm. Supernatant was removed and cartilage washed three times with DPBS, then incubated with trypsin (20 ml/5 g cartilage) for 30 min at 37°C on an orbital shaker. Supernatant was removed and cartilage washed two times with culture medium and incubated with collagenase (3 ml/g cartilage) for 15-20 hr at 37°C on an orbital shaker. The sample was removed from the shaker and the cells allowed to settle for 15 mins, the supernatant was then removed with a sterile 21 gauge needle. The supernatant was centrifuged at 1000rpm for 10 min and the supernatant discarded. The cell pellet was

suspended in 10-20 ml culture medium and the number of cells counted. Cells were seeded into 12-well tissue culture plates at $1.25x10^5$ with 2 ml of culture medium per well or 6-well tissue culture plate at $1.5x10^5$ with 5 ml of culture medium and allowed to reach 90% confluence. Cells were left for 24hr in 1 ml/well (12-well) or 2 ml/well (6-well) of culture medium (serum free) before treatment with cytokines.

2.6 RNA EXTRACTION, REVERSE TRANSCRIPTION AND REALTIME PCR

2.6.1 Purification of RNA

Principle: RNA was purified using a Qiagen RNeasy mini kit (Qiagen Ltd., West Sussex, UK). Samples were first lysed and homogenised in the presence of a highly denaturing guanidinium isothiocyanate containing buffer which immediately inactivates RNases to ensure isolation of intact RNA. Ethanol was added to provide appropriate binding conditions and the sample is then added to an RNeasy mini spin column where the total RNA binds to the membrane and contaminants are washed away. RNA is then eluted in water.

Method

Culture medium was removed from the cells grown in a monolayer. Under RNase free conditions according to the manufacture's instructions, total RNA was extracted from the cells. Briefly, 350 µl of lysis buffer containing 1% β-mercaptoethanol was added per well of a 6-well plate and a scraper used to collect cells. Lysates were then pipetted directly onto a QIAshredder (Qiagen Ltd,. West Sussex, UK) and centrifuged (2 min, 10,000 g). One volume of 70% ethanol was added to the homogenised sample and mixed well by pipetting. The sample was applied to a RNeasy mini spin column for adsorption of RNA to membrane and centrifuged for 15 sec at 10,000 g. The flow through was discarded. Three washes were then preformed to remove contaminants according to the manufacturer's instructions. The purified RNA was eluted with RNase –free water (30 µl). Isolated RNA samples were immediately stored at -80°C. The concentration and purity of RNA was determined following measurement of the absorbance at 260 nm and 280 nm using a Nanodrop.

2.6.2 Reverse transcription

Principle: Reverse Transcription (RT reaction), a process in which single-stranded RNA is reverse transcribed into complementary DNA (cDNA) by using a reverse transcriptase enzyme, primer, dNTPs and RNase inhibitor. The resulting cDNA can be used in RT-PCR reaction. RT reaction is also called first strand cDNA synthesis.

Method

Target RNA was reverse transcribed using the Superscript II reverse transcriptase enzyme Invitrogen™ Life Technologies (Paisley, UK). In the first step, 2 µl (1 µg/µl) random hexamers [pd$(N)_6$] was added to 9 µl of total RNA with a concentration of 1 µg and annealed at 70°C for 10 min. Samples were then chilled on ice. 100 U Superscript II reverse transcriptase (0.5 µl) was added in the presence of 4µl of 1st 5x RT buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl 0.1 M DTT, 0.5 µl 10mM labelled deoxynucleotides (dNTPs) and 0.5 µl of 40 U/µl RNaseOUT and then incubated at 42°C for 1 hr. To minimize variations in reverse transcriptase efficiency, all samples from a single experiment were reverse transcribed simultaneously.

2.6.3 Real-Time PCR

Principle: The Real-Time PCR technique exploits the 5'–3' exonuclease activity of Taq polymerase (Holland et al, 1991) and dual-labelled fluorogenic oligonucleotide probes which emit a fluorescent signal only upon cleavage (Cardullo et al, 1988). These principles are combined in the TaqMan assay system, Applied Biosystems (Warrington, UK). In this system a probe, the so-called TaqMan probe, is designed to anneal to the target sequence between the classical forward and reverse primers. The probe is labelled, with a reporter fluorochrome (e.g., 6-carboxyfluorescein, or FAM) at the 5' end and a quencher dye (e.g., 6-carboxy-tetramethyl-rhodamine, or TAMRA) at the 3' end. Importantly, in its intact form, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The probe has a melting temperature (T_m) approximately 10°C higher than the T_m of the primers, in order to anneal to the amplicon during the extension phase of the PCR process (which is performed at 60°C). Consequently, the probe will be degraded during the extension phase by the 5'–3' exonuclease activity of the Taq polymerase. This will result in an increase in reporter fluorescence emission because reporter and quencher are separated. The amount of fluorescence released is directly

proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of PCR product formation.

Method

The cDNA was diluted for TaqMan reactions; a 2 µl into 200 µl water is required for most genes, with a further 1:5 dilution for 18S. Each reaction uses a 25 µL sample volume, containing 10 µL cDNA sample, 8.333 µl TaqMan universal PCR master mix (2x) (AmpliTaq Gold® DNA Polymerase, AmpErase® UNG, dNTPs with dUTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 10 mM EDTA, 60 nM Passive Reference 1), 2 mM $MgCl_2$, 30 µM of each primer, and 15 µM TaqMan probe. The PCR reactions were performed on the ABI Prism 7900HT SDS, using 96-well microtitre plates in duplicate wells, using the universal temperature cycles: 10 min at 94°C, followed by 35–45 two-temperature cycles (15 s at 94°C and 1 min at 60°C).

Quantification of the results was achieved by production of a standard curve using a cDNA sample known to express the gene of interest, with a top standard of 2.5 µg of cDNA; lower standards were produced by carrying out doubling dilutions of the cDNA. Results were analysed using program SDS 2.2.

2.6.4 TaqMan Low Density Array

Principle: The micro fluidic card functions as an array of reaction vessels for real-time PCR. It consists of a series of 384 interconnecting wells divided into eight sets of assays. Each of the wells contains dried TaqMan primers and probes for one mRNA target. The Micro Fluidic card uses fluorogenic 5' exonuclease assay reagents that detect the real-time amplification of targets and an external endogenous control cDNA. The relative levels of gene expression are determined from the fluorescence data generated during PCR.

Method

The Micro fluidic card was first removed from 4°C and allowed to reach room temperature during the sample preparation. Each sample of cDNA was diluted to between 1 to 100 ng, therefore 2 µl of cDNA was diluted with 48 µl of RNase free water. 50 µl of Taq Man universal PCR master mix (2x) was then added to the diluted cDNA sample, giving a total sample volume of 100 µl. The card was removed from its packaging and 100 µl of sample added to each fill reservoir. The card was then placed inside the Sorvall Micro Fluidic Card bucket and placed inside a Sorvall Legend T centrifuge and spun three times for 1 min at 1200 rpm in order for the sample to fill the card. The plate was removed from the

centrifuge and placed in a Micro Fluidic Card sealer to ensure that the wells of the Micro Fluidic Card are isolated after the sample loading. The plate was then placed in the Real Time PCR machine and gene expression was measured by the machine and analysed using the Relative quantification ΔC_T programme.

2.7 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Principle: Proteins are separated based on size by SDS-PAGE performed under reducing conditions (Laemmli 1970). The polymerisation of acrylamide is initiated by the addition of APS and the base TEMED. TEMED is able to catalyse the decomposition of the persulphate ion producing a free radical which then initiates the polymerisation reaction. Samples are prepared by boiling in β-mercaptoethanol and SDS. β-mercaptoethanol reduces any disulphide bridges and the SDS, an anionic detergent denatures proteins by wrapping around the polypeptide backbone in a ratio of approximately 1.4 grams SDS per gram protein. The bound SDS masks the charge of the proteins, forming anionic complexes with constant net negative charge per unit mass. The purpose of the stacking gel is to concentrate the protein sample into a sharp band before it enters the main separating gel. This is achieved by utilising differences in ionic strength and pH between the electrophoresis buffer and the stacking gel. The stacking gel has a very large pore size, which allows proteins to move freely and concentrate or stack under the effect of the electric field. The band-sharpening effect relies on the fact that negatively charged glycinate ions (in the electrophoresis buffer) have a lower electrophoretic mobility than the protein-SDS complexes, which, in turn have lower mobility than the chloride ions of the loading buffer and the stacking gel. When a current is applied, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical circuit. The protein-SDS complexes concentrate in a very tight band between glycinate and chloride boundaries. Once the glycinate ions reach the separating gel, it becomes more ionised due to higher pH and its mobility increases. The interface between the chloride and glycinate ions leaves behind the protein-SDS complexes, which are left to electrophorese at their own rates. The protein-SDS complexes continue to move towards the anode. Proteins are separated by their size as smaller proteins pass more easily through the pores of the gel. Larger proteins are retarded by frictional resistance due to sieving effects of the gels. After the tracking dye (bromophenol blue) reaches the bottom of the gel, electrophoresis is

stopped. Proteins are fixed into the gel using an acid-methanol mixture and stained using Coomassie Brilliant Blue dye.

Solutions

4x Lower gel buffer (LGB)

1.5M TrisHCl pH 8.8, 0.4% (w/v) SDS.

4x Upper gel buffer (UGB)

0.5M TrisHCl pH 6.8, 0.4% (w/v) SDS.

Upper gel

40% Bis /acrylamide diluted to 4.5% with water and 4x UGB

5x Final sample buffer (FSB)

0.625M TrisHCl pH6.8, 40% (v/v) glycerol, 10% (w/v) SDS, 0.5% (w/v) bromophenol blue, 5% (v/v)β-mercaptoethanol.

10x Running buffer

250mM Tris, 2M glycine, 10% (w/v) SDS.

Coomassie stain

2.5% (w/v) Coomassie Blue G250, 40% (v/v) methanol, 10% (v/v) acetic acid.

Destain

40% (v/v) methanol, 10% (v/v) acetic acid.

Method

Electrophoresis was carried out in a Bio-Rad Mini-Protean II apparatus with 1.0 mm spacers and combs. Polyacrylamide-bis-acrylamide was purchased as a 40% (w/v) (37.5:1 acrylamide:bis) solution, diluted with water and 3 ml LGB to the required percentage (table 2.1). All solutions were allowed to reach room temperature before mixing. Gel mixture (12 ml) was polymerised by the addition of 20 µl TEMED and 60 µl APS (0.2 % w/v) immediately before pouring the gel. The lower gel was overlaid with propan-2-ol to exclude oxygen and to allow polymerisation. Once set, propan-2-ol was washed off the gel and a 4.5 % bis/Acrylamide stacking gel laid on top, combs inserted and the stacker allowed to set. Upper gel (5 ml) was set with 10 µl TEMED and 30 µl 0.2% (w/v) APS. The gel kit was assembled and filled with 1x running buffer. Samples were mixed with 5x FSB and boiled for 5 min. Molecular weight markers were SeeBlue plus. Proteins were electrophoresed at constant 200 V for approximately 40 min (until the dye front reached the end of the gel). Gels were stained for 1 hr with Coomassie Blue and destained. Gels were then photographed using the Syngene Chemigenius II.

Table 2.1 Preparation of SDS-PAGE running gel: volumes for 2 gels.

Percentage gel (%)	40% (w/v) acryl/bis solution (37.5:1) (ml)	dH_2O (ml)	4x LGB (ml)
12.5	3.75	5.25	3.0
10	3.0	6.0	3.0
7.5	2.25	6.75	3.0

2.8 WESTERN BLOTTING

Principle: Proteins were transferred from an SDS-PAGE gel onto nitro-cellulose membrane by means of a current. Proteins bind irreversibly by hydrophobic interactions to the membrane. Blots are then incubated with a protein solution such as non-fat dried milk to block all remaining hydrophobic binding sites on the nitrocellulose membrane. The blot is then incubated with primary antibody, which will bind to the protein of interest. In order to visualise this interaction the blot is then incubated with a secondary antibody with is directed against the IgG of the species that provided the primary antibody. The secondary antibody is labelled with HRP and the method of ECL is used to detect HRP. In the presence of hydrogen peroxide and the chemiluminescent substrate luminal, HRP oxidises the luminal. Following oxidation, the luminal is in an excited state which then decays to ground state via a light emitting pathway. The light emitted is detected by exposing the blot.

Solutions

Transfer Buffer

39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS and 20% (v/v) methanol.

Tris Buffered Saline (TBS)

10 mM Tris HCl pH 7.4, 0.15 M NaCl.

Blocking Buffer

5% (w/v) Marvel in TBS.

Method

Proteins were separated by SDS-PAGE (Section 2.7) and semi-dry blotted onto PVDF membrane essentially according to the method of (Towbin et al. 1979). SeeBlue Plus (Invitrogen) molecular weight makers were used. Proteins were transferred from SDS gels

to PDVF membrane (0.2 μm pore size) and 8 sheets of filter paper were soaked in transfer buffer. A sandwich was made on the anode plate consisting of 4 sheets of filter paper followed by PDVF membrane, the gel and the remaining 4 sheets of filter paper. The sandwich was rolled gently to remove any air bubbles and the cathode plate lowered on top. Transfer was allowed for 1 hr at a current of 80 mA per gel.

Following the transfer, the blot was immersed in blocking buffer for 1 hr at room temperature. The blot was then incubated overnight with primary antibody diluted in TBS/0.05% Tween. The blot was then washed three times in 250 ml TBS/0.05% Tween for 10 min in a large dish on a rocking platform. The blot was then incubated for 1 hr with a secondary antibody conjugated with HRP. The blot was then washed three times in 250 ml TBS/0.05% Tween for 10 min. ECL was carried out according to the manufacter instructions and incubated with the blot for 5 min. ECL reagents were removed using a roller. The blot was then developed using the ABI Gel doc machine for between 1 to 15 min.

2.9 PANARAMA® ANTIBODY MICROARRAY

Principle: Designed for the study of protein expression in cell and tissue extracts, the Panarama® antibody microarray (MPAA3, Sigma Poole, UK) allows the simultaneous identification of multiple proteins and allows the global characterisation of biological samples. The array is used for comparing protein expression profiles of two samples (test versus reference), each sample is labelled with a different Cy™ dye (Cy3 or Cy5) and the two samples are applied simultaneously at equal protein concentrations onto the array. The fluorescent signal intensity for each sample is then recorded individually at the wavelength corresponding to the dye label of the sample and compared. The Panarama® antibody microarray has previously been used for such applications as differentiation of F9 cells (Quackenbush, 2002), breast cancer samples (Kopf et al, 2005) and monitoring human embryonic stem cells pluripotency and viability (Celis et al, 2005).

Solutions

Protease Inhibitor cocktail

Add 300μl of ultrapure water to Protease inhibitor vial (Sigma, P4495). Store the reconstituted solution at -20°C.

Benzonase Working Solution

Benzonase, Ultrapure (Sigma, B8309) supplied as a 50 units/µl solution. For immediate use, prepare a 5 units/µl working solution in the extraction/ labelling buffer (Sigma, E0655) by adding 2 µl of benzonase ultrapure to 18 µl of extraction/ labelling buffer, and store on ice.

Buffer A

10 ml Extraction/labelling buffer (Sigma, E0655), 50 µl reconstituted protease inhibitor cocktail (Sigma, P4495), 100 µl phosphatase inhibitor cocktail 2 (Sigma, P5726), 1.2 ml benzonase working solution (0.6 units/ml). Keep on ice and use immediately.

Washing Buffer

Phosphate Buffered Saline, pH7.4 with TWEEN 20 (Sigma, P3563) dissolved in 1 litre of dH_2O. Filter through a 0.45 µm filter.

Method

Human articular chondrocytes were grown in T75 flasks until they reached 70 – 80 % confluence. The cells were serum starved overnight, before treatment with the appropriate cytokines and inhibitors; the media was then removed from the cells which were then washed twice with ice cold wash buffer. Buffer A was then added to the cells at 1 ml per flask and the cells were then incubated on ice for 5 minutes. The cells were then scraped from the surface of the flask and collected in a microcentrifuge tube. Protein concentration was then assessed using the Bradford assay as described in section 2.11. For labelling the cy3 and cy5 fluorescent dyes (Cy3 monofunctional reactive dye, Amersham Biosciences PA23001; Cy5 monofunctional reactive dye, Amersham Biosciences PA25001) were dissolved in 50–100 ml of carbonate-bicarbonate buffer (pH 9.5–9.6) (Sigma, C3041). For each treatment, 1 ml of extract was used for each dye. The extract was added to each vial and mixed thoroughly; the reaction was incubated at room temperature for 30 minutes whilst ensuring the solution was mixed every 10 minutes. The free Cy3/ Cy5 was removed from the labelled sample by applying it to a sigmaspin column and centrifuged for 2 minutes at 750 x g. A Bradford assay was again carried out to measure the protein concentration of the labelled sample (Y mg/ml). Measurement of the Cy3 and Cy5 absorbance at 552 nm and 650 nm respectively was carried out, using buffer A as a blank. The molar concentration of Cy3 and Cy5 was calculated using the following formula:

$$\text{Cy3 concentration } (\mu M) = A_{552}/0.15$$

$$\text{Cy5 concentration } (\mu M) = A_{650}/0.25$$

The dye to protein molar ratio (D/P ratio) should have a value of >2 and this was calculated according to the manufacturers' instructions in the following manner:

$$\text{Protein } (\mu M) \text{ concentration} = [Y \text{ (mg/ml)} / 60{,}000] \times 10^6$$

$$D/P = \text{Cy3 or Cy5 concentration / Protein concentration of sample}$$

Each array slide was then washed by briefly dipping them in PBS. In a fresh tube 50 µg of each Cy3 and Cy5 labelled sample were mixed together at equal protein concentrations with 5 ml of array incubation buffer (Sigma, A9602) and mixed by inverting the tube. Using the incubation tray provided, the mixture was add to well 1 and the slide immersed in the solution. The tray was protected from light by covering with aluminium foil and incubated for 30mins at room temperature on a rocking platform at a frequency of ~30 rpm. Wash buffer was added to wells 2, 3 and 4 of the tray and the slide carefully removed from well 1 using forceps, and placed into well 2 and incubate for 5 minutes at room temperature whilst shaking on the rocking platform as before. The process was repeated by transferring the slide to wells 3 and 4. The liquid was then decanted from well 4 and replaced with 5 ml of water and the slide was then incubated for a further 2 minutes. The slide was then carefully removed from the tray and allowed to air dry completely for at least 20 minute whilst protecting it from light. The microarray was then scanned using a Axon Genepix Scanner and analyzed using Genepix software.

2.10 NUCLEAR AND CYTOPLASMIC EXTRACTION

Principle: Nuclear and cytoplasmic fraction extraction is a technique used to separate each fraction for cell signalling investigation. A low salt/detergent hypotonic buffer is used to perforate the plasma membrane for cytoplasm protein extraction and a high salt solution is used to leach out nuclear proteins.

Solutions

Hypotonic Buffer

10 mM HEPES pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCL, 1 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.1 % NP40

High Salt Buffer

20 mM HEPES pH 7.9, 420 mM NaCl, 20 % glycerol, 1 mM DTT, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 x protease inhibitor mini tablet per 25 ml of buffer.

Method

Cells grown in T25 flasks were stimulated for the desired time and the media poured off. Cells were washed once in ice-cold PBS and the excess pipetted off. Cells were scraped into 1 ml ice-cold PBS and pelleted at 9660 g for 20 seconds at 4 °C. Cells were resuspended in 100 µl of ice-cold hypotonic buffer and incubated on ice for 15 minutes. Cells were then repelleted as before and the supernatant containing the cytosolic proteins was collected and snap-frozen. The pellet was resuspended in ice-cold hypotonic buffer and supplemented with 0.25 M sucrose. Cells were repelleted as before and the supernatant discarded. The pellet was resuspended in 100 µl of ice-cold high salt buffer and incubated on ice for 30 minutes. The nuclear proteins were then collected by a final centrifugation at 9660 g for 2 minute. The supernatant was collected and snap frozen on dry ice. Both fractions were stored at -80 °C until they were analysed.

2.11 GELATIN ZYMOGRAPHY

Principle: Gelatin zymography is an electrophoretic technique used to identify gelatinase activity in proteins separated in polyacrylamide gels co-polymerised with gelatin. The enzymes are separated under partial denaturing (SDS) but non-reducing conditions, refolded in detergent that removes SDS (Triton X-100) and then incubated overnight in buffer to allow the enzymes to digest the gelatin. Gels are then stained with Coomassie blue and evidence of enzymatic activity is demonstrated by clear bands in a blue background. Both active and pro-enzyme forms of gelatinases may be visualised. The proenzymes are probably activated by gentle denaturation and refolding (Springman et al. 1990). Once refolded into an active confirmation, the proenzymes appear to undergo autolytic self-cleavage in a manner analogous to organomercurial activation (Kleiner and Stetler-Stevenson, 1993). 1,10-phenanthroline, a metalloproteinase inhibitor was used to confirm activity was due to metalloproteinases.

Solutions

Tris/Triton Buffer

20 mM Tris pH 7.8, 2.5% Triton – X100.

Tris/Triton /Ca^{2+}/Zn^{2+} Buffer

20 mM Tris pH 7.8, 10 mM $CaCl_2$, 5 mM $ZnCl_2$, 1% Triton – X100.

5x Final sample buffer

250 mM TrisHCl pH 6.8, 80% (v/v) glycerol, 20% (w/v) SDS, 0.5% (w/v) bromophenol blue.

1,10-Phenanthroline

2 M dissolved in ethanol.

Method

Acrylamide gels (7.5%) co-polymerised with 0.1% (w/v) gelatin were prepared as described in section 2.7 but replacing water with 1.78 mg/ml gelatin. Samples were mixed with 5 x final sample buffer and incubated at room temperature for 30 min. Samples were electrophoresed at 180 V. Gels were washed in Tris/Triton buffer for 2x 1 hr at 4°C ± 1, 10-phenanthroline (2 mM). Gels were then washed in Tris/Triton/Ca^{2+}/Zn^{2+} buffer for 1 hr at 4°C then in the same buffer for 16 hr at 37°C ± 1,10-phenanthroline. Gels were stained with Coomassie Blue G250 for 1 hr and then detained.

2.12 BRADFORD ASSAY

Principle: Based upon the equilibrium between three forms of Coomassie Blue G dye, where under strongly acid conditions, the dyes are most stable in a doubly-protonated red form. However, upon binding to protein, the dyes are most stable as an unprotonated, blue form.

Solutions

Protein Standard

Albumin from bovine serum (2 mg/ml) (Fluka 82515, Sigma)

Bradford Reagent

Coomassie® dye binding protein assay for 1-1,400 µg/ml protein (Sigma B6916)

Method

Remove the Bradford reagent from the fridge and allow it to reach room temperature. Prepare the standards in 0.5 ml eppendorf tubes according to table 2.2, using the appropriate dilution buffer.

To a flat bottom 96-well plate, add 5 µl of the protein standards to separate wells. Add 5 µl of the protein sample to separate wells and add 250 µl of Bradford reagent to each well. Using a plate sealer, seal the plate and place on a shaking platform and allow the samples to mix for 2 minutes and then incubate at room temperature for 30 minutes. After incubation read the plate on a plate reader measuring at absorbance 595 nm. Plot the Net

absorbance vs. the protein concentration of each standard and determine the protein concentration of each unknown sample by comparing the net A_{595} values against the standard curve.

Table 2.2 Protein standard preparation

[BSA] Protein Standard (mg/ml)	Amount of BSA Standard (µl)	Amount of Dilution Buffer (µl)
1.2	120	80
1.0	100	100
0.8	80	120
0.6	60	140
0.4	40	160
0.2	20	180
0.1	10	190
0	0	200

2.13 STATISTICAL ANALYSIS.

The statistical significance was tested for normal distribution and significance between two groups was determined by one-way ANOVA Bonferroni post-hoc test. Values of $p<0.05$ were considered significant.

CHAPTER 3: Effects of Indomethacin and its derivative on cartilage degradation and MMP expression

3.1 INTRODUCTION

During arthritis, the presence of IL-1 is known to play a key role. The expression of IL-1 in articular cartilage is known to induce a catabolic cascade involving cyclooygenase (COX) enzymes. These enzymes have a number of isoforms of which COX-1 and COX-2 are known to catalyse the conversion of arachidonic acid to prostaglandins (PG) producing the proinflammatory product PGE_2 (Xie et al, 1991). These two isoforms do however vary in their expression, COX-1 is the constitutive form of the enzyme, is naturally expressed at low levels and is essential for normal function of many tissues. COX-2 is an inducible isoform which is up regulated following tissue damage, and is therefore often expressed in the cartilage, synovium and synovial fluid of OA joints (Hardy et al, 2002; Vane et al, 1998; Martel-Pelletier et al, 2003). COX inhibitors have been widely used in the management of pain and inflammation for a number of years, with indomethacin a non-steroidal anti-inflammatory drug being used widely in the treatment of autoimmune and chronic inflammatory diseases (Smith et al, 1995). It is a potent non selective cycloxygenase (COX) inhibitor, which is known to inhibit both COX-1 (IC_{50} = 740nM) and COX-2 (IC_{50} = 970nM) (Barnett et al, 1994). NSAIDs such as indomethacin have been reported to regulate the production and secretion of MMPs in both rabbit chondrocytes (Ito et al, 1995) and human monocytes (Corcoran et al, 1992).

Whilst COX inhibitors have clearly been of benefit in inhibiting MMP regulation, they are however associated with gastrointestinal adverse events ranging from dyspepsia to symptomatic and complicated ulcers. The anti-inflammatory and analgesic effects which COX inhibitors have are thought to be due to the inhibition of COX-2 whilst adverse effects appear to be caused by the inhibition of COX-1 (Shi, 2007). With this in mind, a derivative of indomethacin, indomethacin heptyl ester which is known to act as a potent and selective inhibitor of COX-2 (IC_{50} = 40nM) and only has a trivial effect on the activity of COX-1 (IC_{50} > 60 μM) (Kalgutkar et al, 2000), may be a viable alternative to the non-specific COX inhibitor indomethacin, in the treatment of autoimmune and chronic inflammatory diseases

In studies looking at the effect of the COX inhibitor indomethacin in arthritis there has been a great variation in results. In one study it was shown that the IL-1 stimulated

induction of TIMP-1 and proMMP-1 in rheumatoid synovial fibroblast was blocked by indomethacin suggesting a role for prostaglandins in the inhibition of TIMP-1 and proMMP-1 (Takahashi et al, 1997). Indomethacin has also been shown to suppress TIMP-1 and proMMP-1 in pathological tissues from rheumatoid arthritis patients (Krane et al, 1990). However, in other studies indomethacin has been shown to enhance IL-1 and TNF-α-induced production of MMP-1 in rheumatoid synovial fibroblasts (Di Battista et al, 1994; Takahashi et al, 1997). In another paper indomethacin inhibited MMP production by TNF-α but this was not seen with IL-1 where neither inhibition nor induction was seen (Syggelos et al, 2006).

In studies using various cell types, it has been shown that the effect of indomethacin on prostaglandin (PGE) levels causes PGE-induced cyclic adenosine monophosphate (cAMP) to down regulate IL-1 induced synthesis of MMPs in human uterine cervical fibroblasts (Takashasi et al, 1991). In human macrophages indomethacin has been seen to suppress agonist-induced production of TIMP and collagenase (Pentland et al, 1995). In another study however, indomethacin up regulated PGE2 and MMP-1 mRNA levels in human dermal fibroblasts (Mauviel et al, 1994). Therefore the effect of indomethacin on TIMPs and MMPs appears to vary with cell types and experimental system.

3.2 AIMS

The aims of this chapter are to investigate the effect of cycloxygenase inhibitors indomethacin and its derivative indomethacin heptyl ester on cartilage collagen breakdown.

3.3 METHODS

3.3.1 Bovine nasal cartilage degradation assay

In order to study the effects of indomethacin and its derivative indomethacin heptyl ester on the degradative processes of cartilage breakdown, discs of bovine cartilage were incubated with IL-1α [1 ng/ml] + OSM [10 ng/ml] either alone or in the presence of indomethacin [0.4 – 50 μM] or indomethacin heptyl ester [5 – 500nM] for 14 days. IL-1α when used in combination with OSM can be used as a positive control for both

proteoglycan and collagen degradation in the explant model, reproducibly inducing the synergistic release of both proteoglycan and collagen. Supernatants were harvested at day 7, and cartilage discs were restimulated with identical conditions for a further 7 days. On day 14, supernatants were harvested and the remaining cartilage digested. The amount of proteoglycan and collagen released into the media was calculated as a mean percentage of the total, and collagenolytic activity calculated as units/ ml. The concentrations for the dose range of each individual drug was selected in order to exceed the IC_{50} values and also taking into account concentrations used in previous studies.

3.3.2 MMP and TIMP ELISAs

Primary human articular chondrocytes were extracted from donated human articular cartilage as described in section 2.5. Cell were incubated with the combination of IL-1α [0.2 ng/ml] + OSM [10 ng/ml) alone, or in the presence of either indomethacin [2 – 50 μM] or indomethacin heptyl ester [5 – 500 nM]. Supernatants were then harvested after 24hr and assayed for MMP-1, MMP-13 and TIMP-1 by ELISA as described in section 2.4.

3.3.3 Real Time PCR

Extracted primary human articular chondrocytes were incubated with the combination of IL-1α [0.2 ng/ml] + OSM [10 ng/ml) alone, or in the presence of either indomethacin [2 – 50 μM] or indomethacin heptyl ester [5 – 500 nM]. RNA was extracted from the cells, reverse transcribed and measured for gene expression as described in section 2.6.

3.3.4 Gelatin Zymography

Primary human articular chondrocytes were incubated with and without the combination of IL-1α [0.2 ng/ml] + OSM [10 ng/ml) alone, or in the presence of either indomethacin [2 – 50 μM] or indomethacin heptyl ester [5 – 500 nM]. Supernatants were then harvested after 24hr and assayed by gelatin zymography for the activity of MMP-2 and –9 as detailed in section 2.9.

3.3.5 Preparation of Indomethacin and Indomethacin heptyl ester

Indomethacin and indomethacin heptyl ester were made up to stock concentrations of 1mM and 100 μM respectively in ethanol, and further diluted to assay concentrations in condition media.

3.4 RESULTS

3.4.1 The effect of adding indomethacin to resorbing bovine nasal cartilage in explant culture

In order to determine the effect of the COX inhibitor indomethacin on cartilage degradation, indomethacin was added to bovine nasal cartilage chips stimulated to resorb with IL-1α + OSM.

3.4.1.1 The effect on proteoglycan degradation of adding indomethacin to resorbing bovine nasal cartilage in explant culture

Indomethacin was added to bovine nasal cartilage stimulated to resorb with IL-1α + OSM. In the experiment shown, 80.7 ± 4.4% proteoglycan release was measured at day 7 (figure. 3.1 a) from IL-1α + OSM treated cartilage and by day 14 of culture, 99.8 ± 0.1% of proteoglycan was release. The addition of indomethacin [0.4 – 50 μM] to IL-1α + OSM treated bovine nasal cartilage resulted in only a small decrease in proteoglycan release at the highest concentration of indomethacin (50 μM) to 67.6 ± 5.6%, whilst no effect was seen with the low doses of indomethacin by day 7. By day 14, indomethacin failed to have an effect on the resorbing bovine nasal cartilage. These data suggests that whilst there was a slight drop in proteoglycan release from the resorbing cartilage with the addition of indomethacin, indomethacin does not significantly affect proteoglycan release when cartilage is treated with these concentrations of IL-1 + OSM.

3.4.1.2 The effect of adding indomethacin to resorbing bovine nasal cartilage in explant culture on the release of collagen fragments

Bovine nasal cartilage was stimulated to resorb with IL-1α + OSM. At day 7, 2.2 ± 0.3% collagen was released from the stimulated cartilage. By day 14, 97.0 ± 0.9% (figure 3.2 a) of collagen fragments had been released from the resorbing cartilage into the medium. By adding indomethacin to IL-1α + OSM treated bovine nasal cartilage, there was no effect on collagen release at day 7, however by day 14 the release of collagen from the resorbing cartilage had significantly decreased with the addition of indomethacin at 10 and 50 μM to 79.0 ± 2.1% and 5.8 ± 1.2% respectively, whilst lower doses of indomethacin showed no effect on resorbing bovine nasal cartilage. This suggests that indomethacin has the ability to block the release of collagen fragments from resorbing bovine nasal cartilage and therefore prevent the breakdown of cartilage.

Figure 3.1 The effect of adding Indomethacin to IL-1α + OSM treated bovine nasal cartilage on the release of proteoglycan. Bovine nasal cartilage discs were cultured in medium IL-1α (1ng/ml) + OSM (10ng/ml) ± indomethacin (0.4, 2, 10 and 50μM), ± Medium was removed and replenished with identical reagents at day7. At day14, medium was removed and the remaining cartilage digested with papain. As a measure of proteoglycan release in day 7 and 14 media, and cartilage digests were assayed. **a)** Proteoglycan release by day 7 was calculated as a % of total proteoglycan. Results are expressed a mean ± SD.

a) Day 7 proteoglycan release

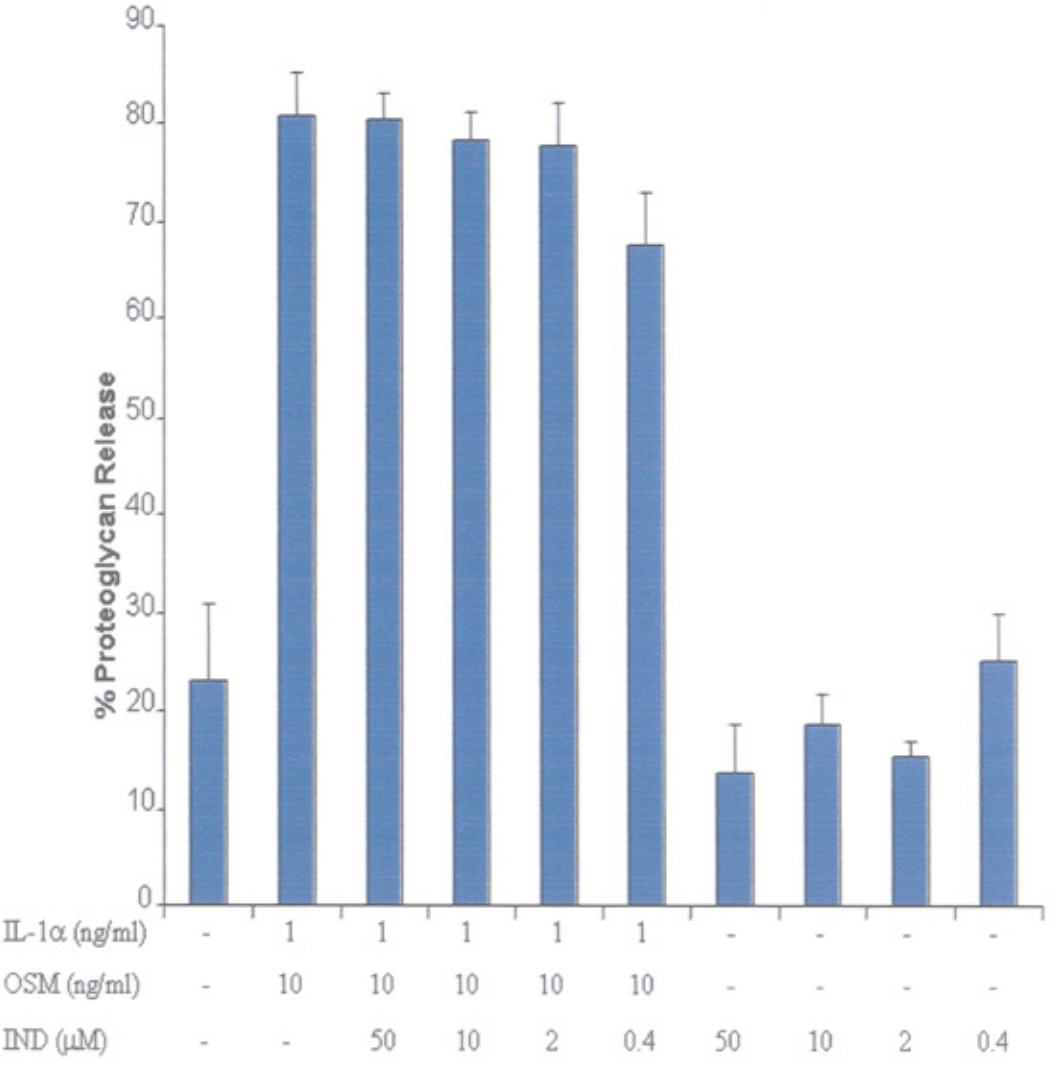


Figure 3.2 The effect of adding Indomethacin to IL-1α + OSM treated bovine nasal cartilage on the release of collagen. Bovine nasal cartilage discs were cultured in medium IL-1α (1ng/ml) + OSM (10ng/ml) ± indomethacin (0.4, 2, 10 and 50μM). Medium was removed and replenished with identical reagents at day7. At day14, medium was removed and the remaining cartilage digested with papain. As a measure of collagen release in day 7 and 14 media, and cartilage digests were assayed. **a)** Total collagen release (day 7 + day 14) was calculated as a % of total collagen. Results are expressed a mean ± SD. ***, P=0.001

a) Total collagen release (day 7 + day 14)

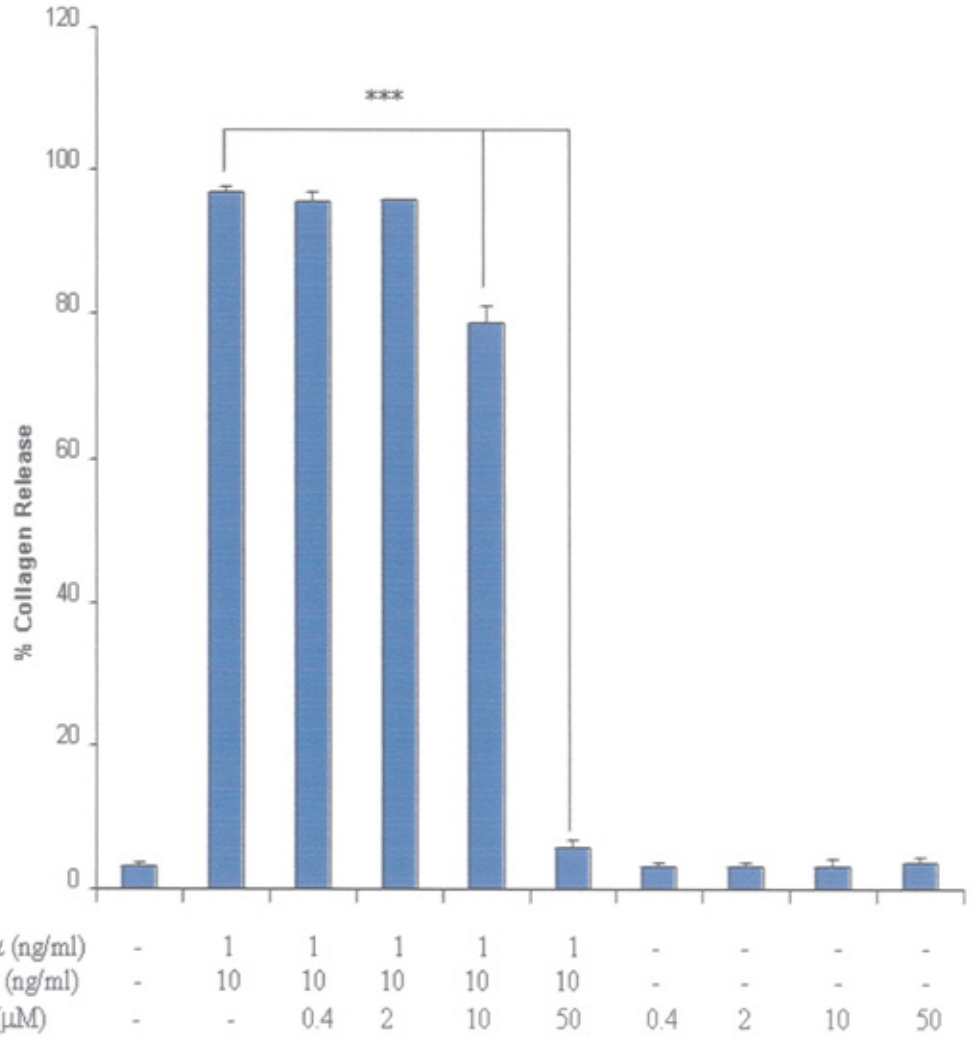
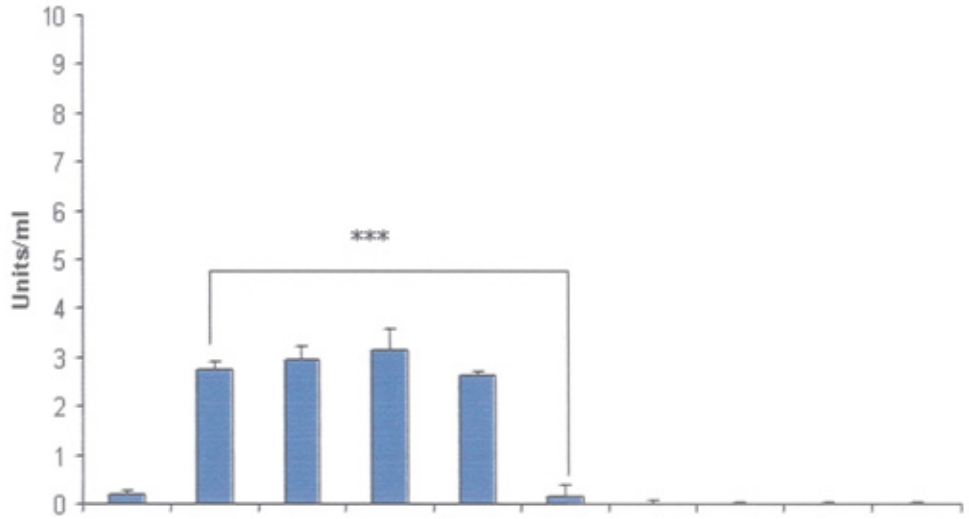
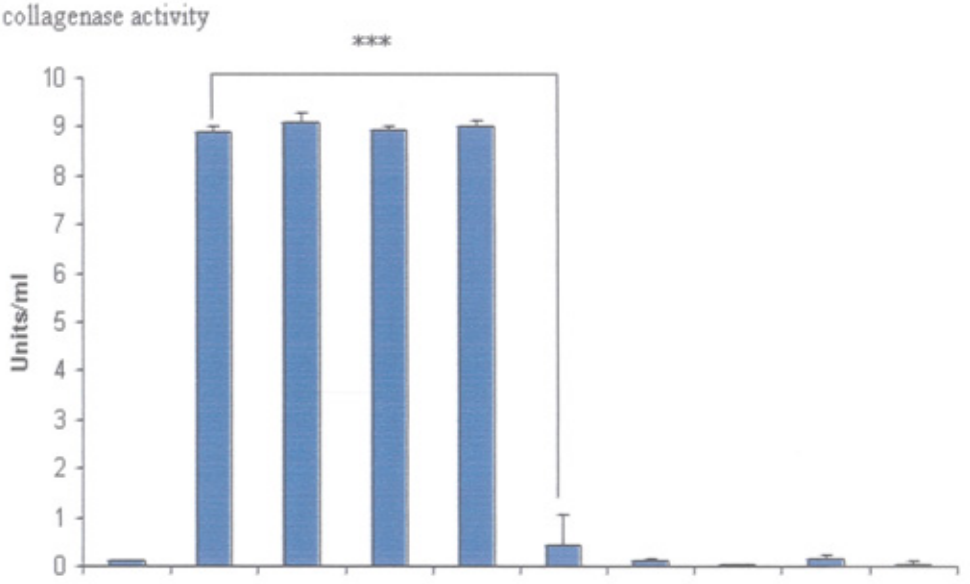


Figure 3.3 Indomethacin inhibits IL-1 + OSM induced collagenolytic activity from cartilage. Bovine nasal cartilage was treated as described in figure 3.2. The levels of (a) active and (b) total collagenolytic activity in the media removed from bovine nasal cartilage culture at day 14 after stimulation with IL-1 + OSM (with and without indomethacin) were measured as described in Materials. Results shown are expressed in units/ml mean ± SD. *** = $P<0.001$

a) Active collagenase activity



b) Total collagenase activity



IL-1α (ng/ml)	-	1	1	1	1	1	-	-	-	-
OSM (ng/ml)	-	10	10	10	10	10	-	-	-	-
IND (μM)	-	-	0.4	2	10	50	0.4	2	10	50

3.4.1.3 The effect of indomethacin on the release of collagenase activity from resorbing bovine nasal cartilage in explant culture

Levels of active and total (pro + active) collagenase activities were measured in day 7 and 14 media from bovine nasal cartilage explants in culture. Explants were treated with IL-1α and OSM, with and without the presence of indomethacin. Figure 3.3 shows the collagenolytic activity within the media from the collagen release experiment shown in figure 3.2. The level of collagenase activity in the day 7 media was undetectable (data not shown). In day 14 media the levels of active collagenase released from IL-1α + OSM stimulated cartilage compared to the control media showed an increase from control values of 0.2 ± 0.1 units/ml to 2.8 ± 0.2 units/ml. With the addition of indomethacin to IL-1α + OSM stimulated cartilage, a significant reduction was seen in the level of active collagenase released into the media with the highest dose of indomethacin reducing levels to 0.4 ± 0.6 units/ml (figure 3.3a). The addition of APMA to the media activates the pro collagenases allowing the measurement of total collagenase activity levels. Whilst the APMA did not increase the level of total collagenase in the control sample, the amount of activity in the IL-1α + OSM stimulated cartilage media increased to 8.9 ± 0.2 units/ml. The addition of indomethacin to the resorbing cartilage resulted in a significant reduction in collagenolytic activity with the 50 μM dose to 0.4 ± 0.6 unit/ml, however lower doses of indomethacin failed to have an effect on collagenase activity. The increase in both active and total collagenase activity which was seen when the bovine nasal cartilage explants were stimulated to resorb with IL-1α + OSM demonstrates the powerful degradative effects of these cytokines in combination as described previously (Cawston et al. 1995). The effect seen in this experiment with the addition of indomethacin, suggests that indomethacin at a dose of 50 μM is able to block the effect of IL-1α + OSM on the collagenolytic activity released by cartilage during the breakdown process.

3.4.2 The effect of indomethacin heptyl ester on resorbing bovine nasal cartilage in explant culture

In order to determine the effect of indomethacin heptyl ester, which is known to be highly specific for COX-2 on the processes of cartilage degradation, this compound was added to bovine nasal cartilage chips stimulated to resorb with IL-1α + OSM.

3.4.2.1 The effect of adding indomethacin heptyl ester to resorbing bovine nasal cartilage in explant culture on proteoglycan degradation

The indomethacin derivative, indomethacin heptyl ester [5 – 500 nM] was added to bovine nasal cartilage stimulated to resorb with IL-1α + OSM. At day 7, 52.4 ± 17.3% proteoglycan release was measured at day 7 (figure. 3.4a) from IL-1α + OSM treated cartilage, the addition of the indomethacin derivative to IL-1α + OSM treated bovine nasal cartilage showed no effect on proteoglycan release.

Proteoglycan release into the culture medium by day 14, showed a release of 83.1 ± 9.5% with IL-1α + OSM in combination (figure 3.4b). The addition of the derivative to IL-1α + OSM treated bovine nasal cartilage had no effect on proteoglycan release with the derivative at a concentration of 500 nM. The data suggests that indomethacin heptyl ester has no effect on the release of proteoglycan from cartilage, when treated the above concentrations of cytokines.

3.4.2.2 The effect of indomethacin heptyl ester on the release of collagen fragments from resorbing bovine nasal cartilage explants

Bovine nasal cartilage was stimulated to resorb with IL-1α + OSM with and without indomethacin heptyl ester. At day 7, 2.8 ± 0.4% (figure 3.5a) collagen was released from the stimulated cartilage compared to the control 0.9 ± 0.1%. The addition of the indomethacin derivative caused a small, but not significant increase at all three concentrations, the highest level being 6.3 ± 0.5%. By day 14, the percentage collagen release from the resorbing cartilage into the medium had increased to 89.1 ± 6.8% (figure 3.5b), compared to a release of collagen from the control of 1.7 ± 0.2%. By adding the indomethacin heptyl ester to IL-1α + OSM treated bovine nasal cartilage, there was a small but significant increase in the release of collagen fragments into the media at doses of 5, 50 and 500 nM of 95.3 ± 1.0%, 95.6 ± 0.4% and 96.3 ± 0.5% respectively. This suggests that the COX-2 specific inhibitor indomethacin heptyl ester causes an increase in the release of collagen fragments from resorbing bovine nasal cartilage. If these effects are reproduced in vivo this could result in an increase in joint damage during the process of cartilage breakdown.

3.4.2.3 The effect of indomethacin heptyl ester on the release of collagenase activity from resorbing bovine nasal cartilage in explant culture

Figure 3.4 The effect of adding Indomethacin heptyl ester to IL-1α + OSM treated bovine nasal cartilage on the release of proteoglycan. Bovine nasal cartilage discs were cultured in medium IL-1α (1ng/ml) + OSM (10ng/ml) ± indomethacin heptyl ester (5, 50 and 500nM). Medium was removed and replenished with identical reagents at day 7. At day 14, medium was removed and the remaining cartilage digested with papain. As a measure of proteoglycan release in day 7 and 14 media, and cartilage digests were assayed. **a)** Proteoglycan release by day 7 was calculated as a % of total proteoglycan. Results are expressed a mean ± SD.

a) Proteoglycan release day 7

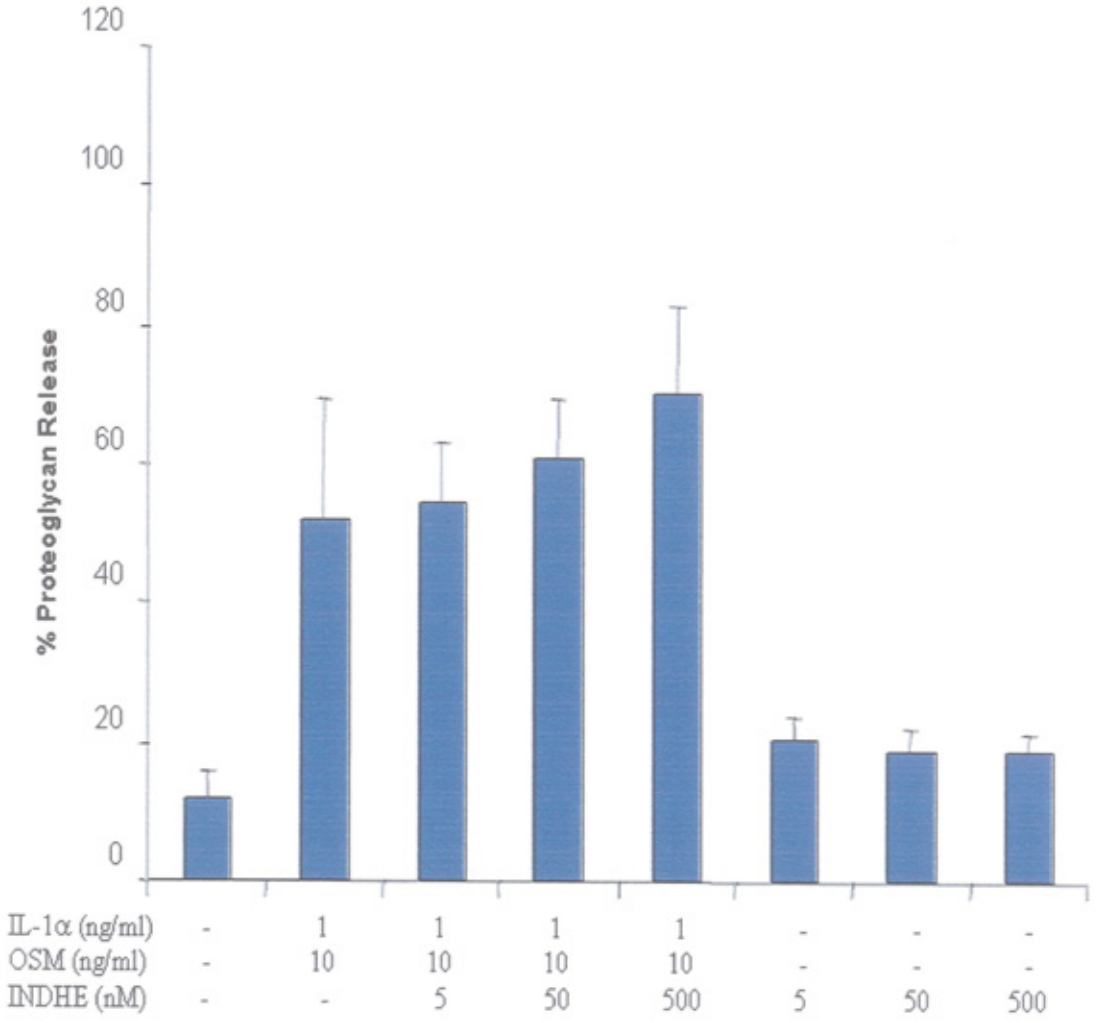


Figure 3.5 The effect of adding Indomethacin heptyl ester to IL-1α + OSM treated bovine nasal cartilage on the release of collagen. Bovine nasal cartilage discs were cultured in medium IL-1α (1ng/ml) + OSM (10ng/ml) ± indomethacin heptyl ester (5, 50 and 500nM). Medium was removed and replenished with identical reagents at day 7. At day 14, medium was removed and the remaining cartilage digested with papain. As a measure of collagen release in day 7 and 14 media, and cartilage digests were assayed. **a)** Total collagen release (day 7 + day 14) was calculated as a % of total collagen. Results are expressed a mean ± SD. *, P=0.05 **,P=0.01

a) Total collagen release (day 7 + day 14)

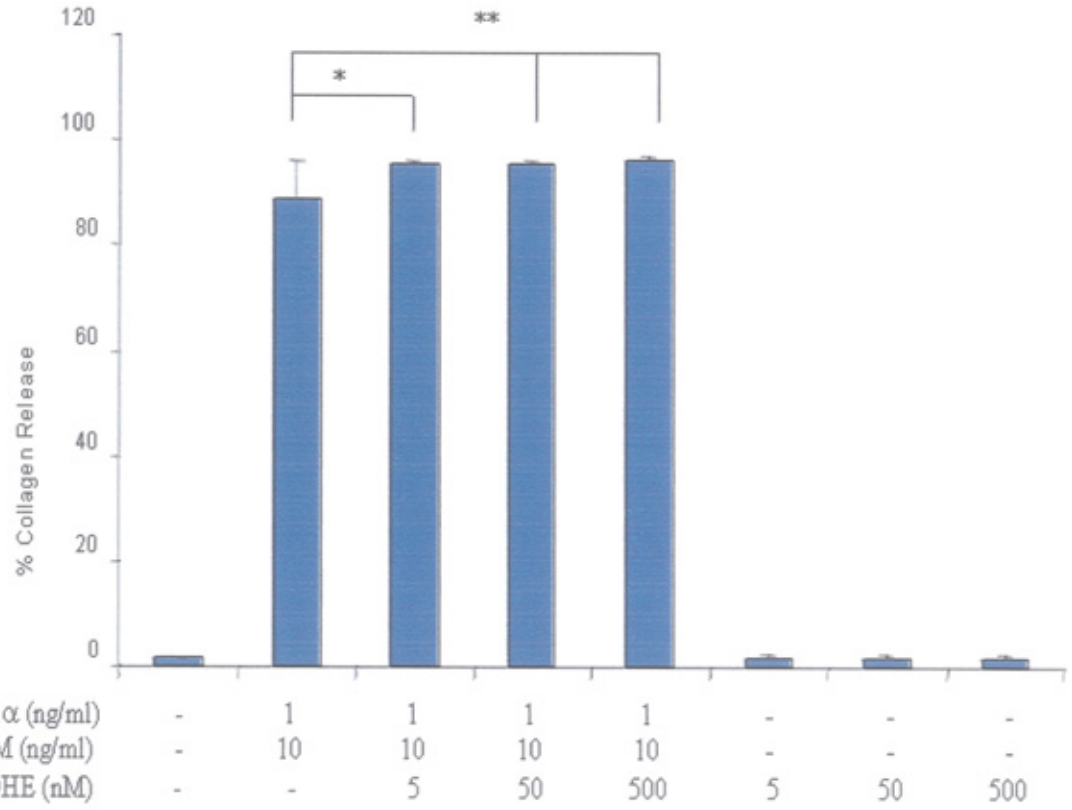
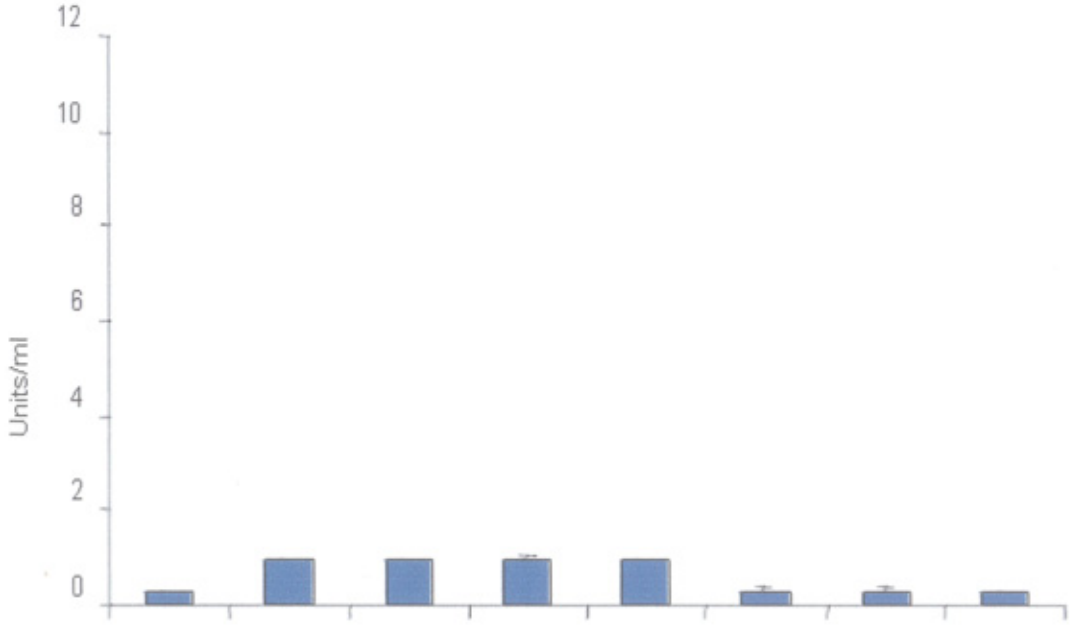
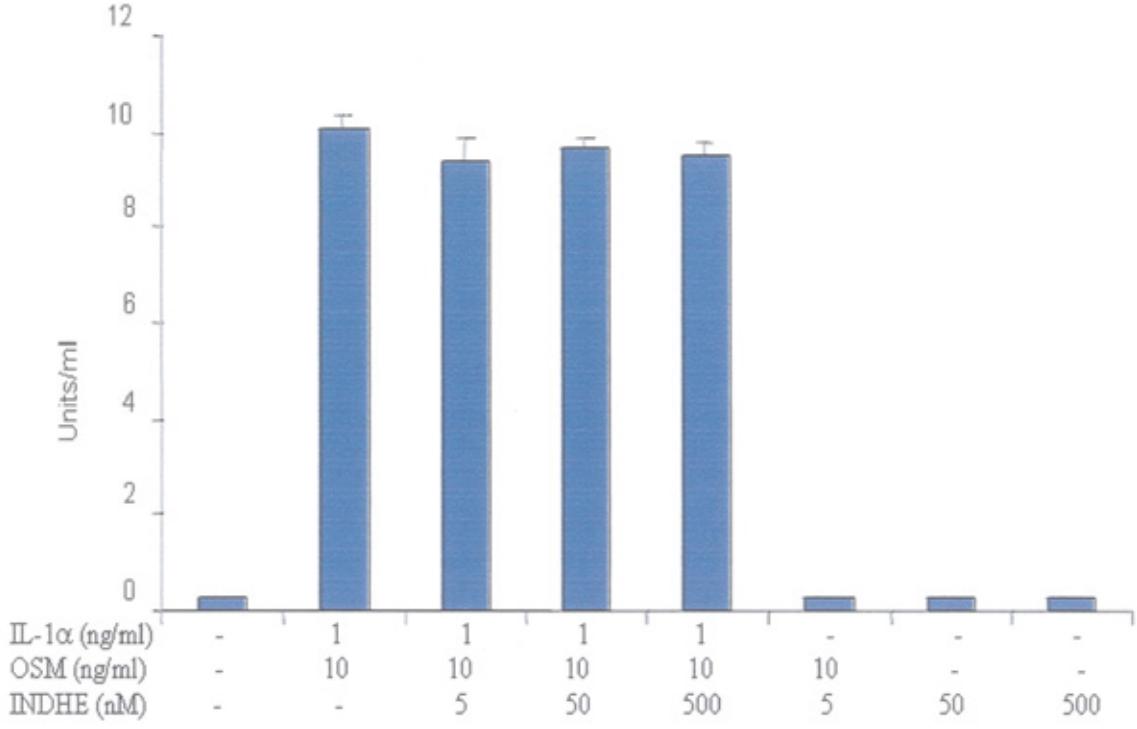


Figure 3.6 The effect of Indomethacin heptyl ester on IL-1 + OSM induced collagenolytic activity from cartilage. Bovine nasal cartilage was treated as described in figure 3.4. The levels of (a) active and (b) total collagenolytic activity in the media removed from bovine nasal cartilage culture at day 14 after stimulation with IL-1 + OSM (with and without indomethacin heptyl ester) were measured as described in Materials. Results shown are expressed in units/ml mean ± SD.

a) Active collagenase activity



b) Total collagenase activity



Day 7 and 14 conditioned media from IL-1α + OSM stimulated cartilage with and without indomethacin heptyl ester from the experiment shown in figures 3.4 and 3.5 was assayed to measure the release of active and total (pro + active) collagenase activity. Collagenase activity levels from the day 7 media were too low and below the limit of detection of the assay (data not shown).

In day 14 media the levels of active collagenase released from IL-1α + OSM stimulated cartilage compared to the control media showed an increase from 0.24 ± 0.01 units/ml to 0.97 ± 0.03 units/ml. With the addition of indomethacin heptyl ester to IL-1α and OSM stimulated cartilage, no significant increase was seen in the levels of collagenase activity released into the media from the resorbing cartilage (figure 3.6a).

The activation of pro collagenases by the addition of APMA to the media allowed the measurement of total collagenase activity to take place. No change was seen in the control media with the addition of APMA, whilst after IL-1α + OSM stimulation there was an increase in total collagenase levels of 10.1 ± 0.2 units/ml. The addition of the indomethacin derivative to the resorbing cartilage had no effect on inhibiting total collagenolytic activity (figure 3.6b). These data suggests that indomethacin heptyl ester has no effect on the levels of both total and active collagenases by IL-1α + OSM.

3.4.3 The effect of both indomethacin and its derivative indomethacin heptyl ester on the release of MMP-1 and -13, and TIMP-1 into the media from IL-1α, and IL-1α + OSM stimulated human articular chondrocytes

In order to determine the effect of the non-specific COX inhibitor indomethacin and its COX-2 specific derivative indomethacin heptyl ester on the release of MMP-1 and -13 and TIMP-1 from cells in culture, both indomethacin and its derivative were added to human articular chondrocytes (HACs) which had been stimulated to resorb with cytokines IL-1α + OSM.

3.4.3.1 The effect of indomethacin and indomethacin heptyl ester on MMP-1 levels in the media from IL-1α, and IL-1α + OSM stimulated human articular chondrocytes

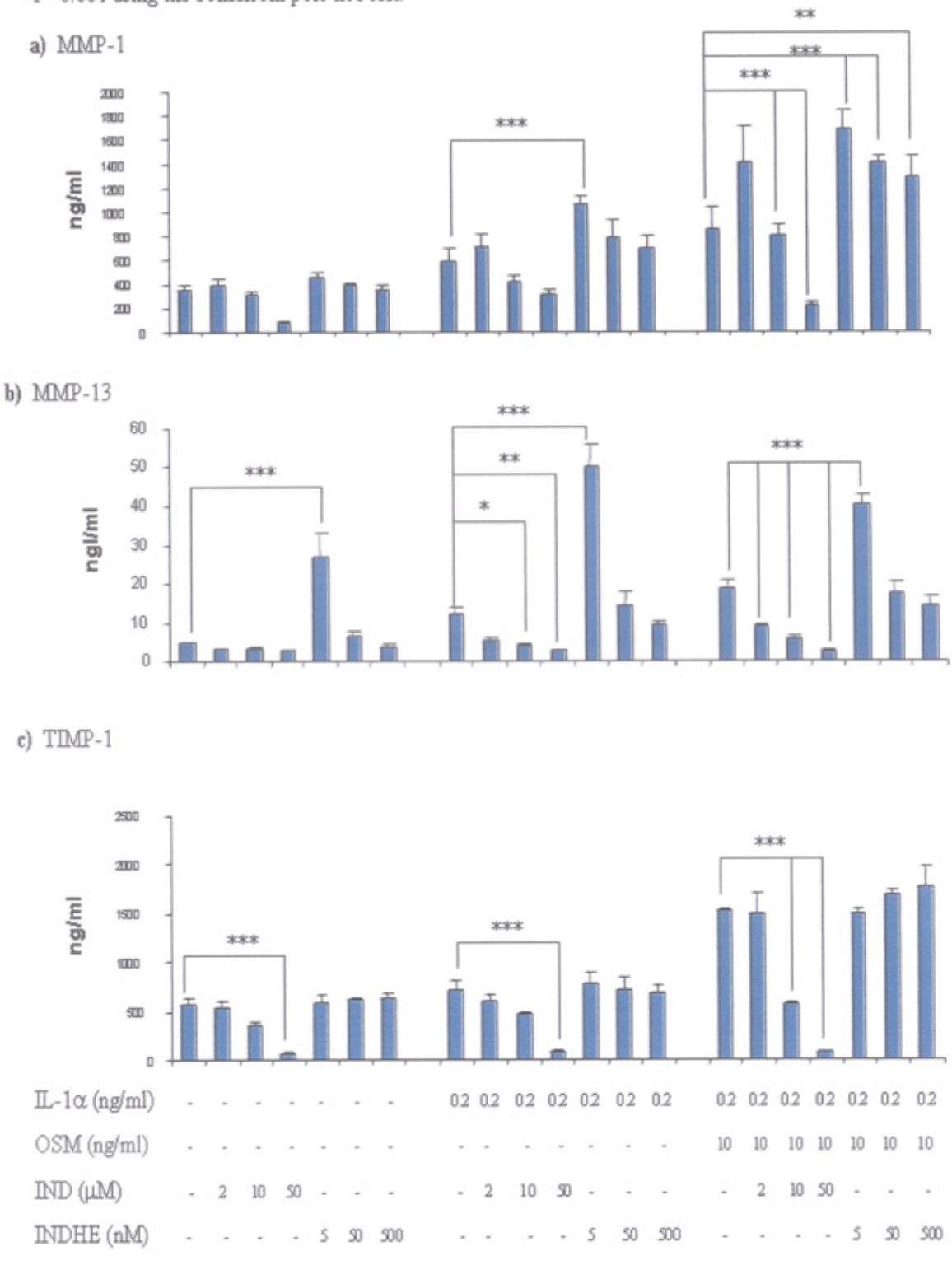
Human articular chondrocytes digested from cartilage donated from hip and knee replacement operations were allowed to grow to 80% confluence. The cells were serum starved overnight and were then stimulated with and without IL-1α, and IL-1α + OSM ± indomethacin or its derivative indomethacin heptyl ester for 24hr, after which the media

was harvested and assayed for MMP-1. Media from control samples showed a release of MMP-1 into the media of 362.4 ± 31.9 ng/ml (Figure 3.7a). After stimulation with IL-1α and IL-1α + OSM in combination the levels of MMP-1 in the conditioned HAC medium were 595.3 ± 100.6 ng/ml and 865.1 ± 177.9 ng/ml respectively. With the addition of indomethacin, cells treated with IL-1α alone showed a slight but not significant reduction in the level of MMP-1 release with indomethacin at a dose of 10 and 50 μM, with indomethacin at 2 μM a slight increase was seen though this result was not significant compared to IL-1α alone. When indomethacin was added to IL-1α + OSM treated HACs, indomethacin at the higher two concentrations of 10 and 50 μM showed a significant decrease in the release of MMP-1 from the cell of 810.1 ± 88.3 ng/ml and 220.2 ± 28.9 ng/ml respectively. With indomethacin heptyl ester added to IL-1α alone, no change was seen with the two higher doses of the derivative, but with the lowest dose of the derivative (5 nM) there was a significant increase in MMP-1 levels. With indomethacin heptyl ester added to IL-1α and OSM in combination there was a significant induction of MMP-1 release with all concentrations from 5 to 500 nM of 1686.9 ± 151.9, 1402.7 ± 62.0 and 1287.7 ± 172.1 ng/ml respectively. Interestingly, indomethacin heptyl ester gave a greater induction of MMP-1 with the lowest dose.

3.4.3.2 The effect of indomethacin and indomethacin heptyl ester on MMP-13 levels media from IL-1α, and IL-1α and OSM stimulated human articular chondrocytes

Media harvested from the experiment described in section 3.4.3.1 was also assayed for MMP-13 release. Media from the control sample contained 5.6 ± 0.1 ng/ml of MMP-13 (figure 3.7b), after stimulation with IL-1α, and IL-1α + OSM in combination the level of MMP-13 released into the media by the cells was 12.4 ± 1.7 ng/ml and 19.0 ± 2.1 ng/ml respectively. The addition of indomethacin alone to the cells showed no change in MMP-13 expression. When indomethacin is added to cells treated with IL-1 α, MMP-13 expression was reduced significantly with doses of 10 and 50 μM, the highest concentration reducing MMP-13 release to 2.8 ± 0.1 ng/ml. The addition of indomethacin to IL-1α and OSM treated cells showed a significant decrease in MMP-13 release at all three concentrations; 2 μM reduced MMP-13 levels to 8.9 ± 0.6 ng/ml, 10 μM reduced levels to 6.1 ± 0.6 ng/ml and 50 μM reducing levels of MMP-13 to 2.8 ± 0.01 ng/ml from 19.0 ± 2.1 ng/ml. When cells were treated with indomethacin heptyl ester there was a significant up regulation in the release of MMP-13 compared to the control levels and at 5

Figure 3.7 Effect of Indomethacin and its derivative on MMP-1, MMP-13 and TIMP-1 levels released from human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.2ng/ml), and IL-1 (0.2 ng/ml) + OSM (10ng/ml) in combination for 24 hours in the presence of Indomethacin at 2, 10 and 50μM and Indomethacin heptyl ester at 5, 50 and 500 nM. The media was harvested after 24 hours and assayed for (a) MMP-1, (b) MMP-13 and (c) TIMP-1 by ELISA as previously described. The results shown are expressed as ng/ml (mean ± SD). Statistical significance was assessed where **P*<0.05, ***P*<0.005 , ****P*<0.001 using the bonferroni post-hoc test.



nM MMP-13 levels were increased to 27.1 ± 6.1 ng/ml from 5.2 ± 0.1 ng/ml. However, this effect was not seen with indomethacin heptyl ester at the two higher concentrations. The addition of indomethacin heptyl ester to cells stimulated with IL-1α alone showed no change at doses 500 and 50 nM, whilst at a dose of 5 nM indomethacin heptyl ester increased the production of MMP-13 by the IL-1α stimulated cells to 50.1 ± 5.5 ng/ml from 12.4 ± 1.7 ng/ml with IL-1 alone. Similarly, indomethacin heptyl ester when added at 5 nM to cells treated with IL-1 + OSM increased the levels of MMP-13 to 40.2 ± 2.7 ng/ml from 19.0 ± 2.1 ng/ml in the presence of IL-1α + OSM alone. The indomethacin heptyl ester had no effect at the higher two concentrations.

3.4.3.3 The effect of indomethacin and indomethacin heptyl ester on the levels of TIMP-1 released into the media from IL-1α, and IL-1α + OSM stimulated human articular chondrocytes

Conditioned media from the experiment as used in sections 3.4.3.1 was assayed to measure the levels of TIMP-1 released from the cells into the media (figure 3.7c). TIMP-1 levels in the control samples were 574.4 ± 64.4 ng/ml; after stimulation with IL-1α, and IL-1α + OSM the TIMP-1 levels increased to 718.3 ± 93.9 and 1528.9 ± 6.4 ng/ml respectively. The addition of indomethacin to the cells caused TIMP-1 levels to be significantly reduced, at the highest dose (50 μM) to 69.6 ± 7.2 ng/ml from 574.4 ± 64.4 ng/ml, lower doses showed no effect. Similarly, when indomethacin was added to IL-1α treated cells, indomethacin at 50 μM significantly reduced TIMP-1 levels from 718.3 ± 93.0 ng/ml to 88.0 ± 4.2 ng/ml. The lower doses of indomethacin had no effect on TIMP-1 expression by the IL-1α treated HACs. The addition of indomethacin to IL-1α + OSM stimulated HACs, significantly reduced TIMP-1 levels at concentrations of 10 and 50 μM, to 562.2 ± 26.6 and 81.2 ± 8.3 ng/ml respectively from 1528 ± 6.4 ng/ml. Indomethacin at 2 μM had no effect. Indomethacin heptyl ester had no significant effect on TIMP-1 levels when added to cells treated with control media, IL-1α alone or IL-1α + OSM. These data suggest that indomethacin may target a pathway which is not affected by indomethacin heptyl ester.

3.4.4 The effect of both indomethacin and its derivative indomethacin heptyl ester on the gene expression of MMP-1 and -13, and TIMP-1 in human articular chondrocytes stimulated with IL-1α, and IL-1α + OSM

It is important to determine the effect of indomethacin and its COX-2 specific derivative, indomethacin heptyl ester, on gene expression levels of MMP-1 and -13 and TIMP-1. Both indomethacin and its derivative were added to human articular chondrocytes (HACs) which had been stimulated with cytokines IL-1α and IL-1α + OSM as described in section 3.4.3, and cells were lysed to extract RNA in order to study changes in gene expression.

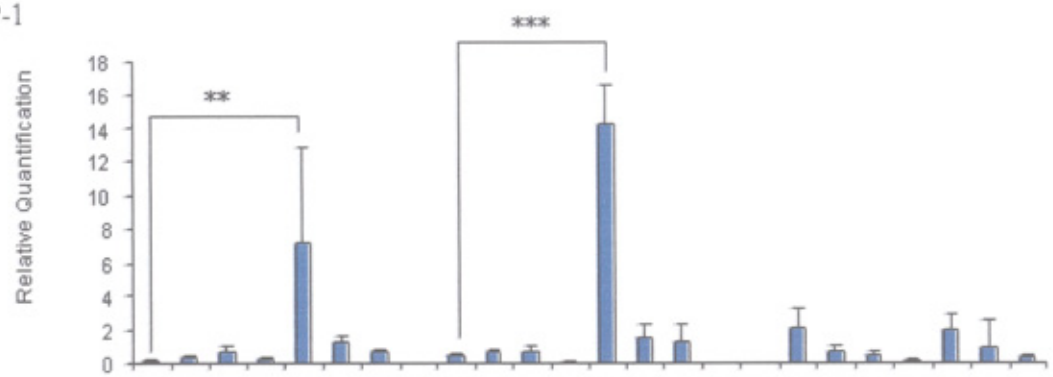
3.4.4.1 The effect of indomethacin and indomethacin heptyl ester on MMP-1 gene expression levels in human articular chondrocytes stimulated with IL-1α, and IL-1α + OSM

Human articular chondrocytes digested from donated knee cartilage were allowed to grow to 80% confluence. The cells were serum starved overnight and were then stimulated with and without IL-1α, and IL-1α + OSM ± indomethacin or its derivative indomethacin heptyl ester for 24hr, after which the cells were lysed and RNA extracted using Qiagen RNeasy kits before being reverse transcribed into cDNA for analysis of MMP-1 by Real-Time PCR. Results are expressed as relative quantification (RQ) after being normalised to 18s (figure 3.8a). MMP-1 levels from cDNA from cells with no treatment (control) were measured to be 0.17 ± 0.04 RQ; after stimulation with IL-1α, and IL-1α + OSM the amount of MMP-1 was seen to be 0.49 ± 0.14 RQ and 2.12 ± 1.16 RQ respectively. Cells treated with indomethacin alone showed no change in MMP-1 levels compared to control. Treatment with indomethacin heptyl ester alone, at 5 nM showed a significant increase in MMP-1 levels to 7.2 ± 2.28 RQ from 0.2 ± 0.04 RQ. The addition of indomethacin to cells treated with IL-1α had no effect on MMP-1 levels, whilst with indomethacin heptyl ester (5 nM) caused a significant increase in MMP-1 expression at the gene level; higher doses of indomethacin heptyl ester had no effect. When indomethacin was added to cells treated with IL-1α + OSM all three concentrations showed no significant change in levels of MMP-1. The addition of indomethacin heptyl ester to the IL-1α and OSM treated cells showed no effect on the expression. This suggests that indomethacin heptyl ester at 5 nM may affect an IL-1α cell signalling pathway such as AKT or NF-kB causing the up regulation of MMP-1 expression from the cells.

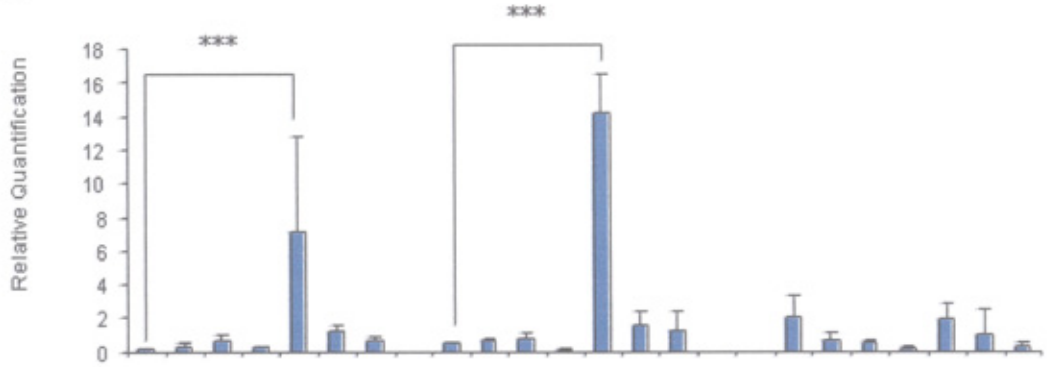
3.4.4.2 The effect of indomethacin and indomethacin heptyl ester on MMP-13 gene expression levels in human articular chondrocytes stimulated with IL-1α, and IL-1α + OSM

Figure 3.8 Effect of Indomethacin and its derivative on the gene expression levels of MMP-1, MMP-13 and TIMP-1. Human articular chondrocytes were stimulated with IL-1 (0.2ng/ml), and IL-1 (0.2ng/ml) + OSM (10ng/ml) in combination for 24 hours in the presence of Indomethacin at 2, 10 and 50μM and Indomethacin heptyl ester at 5, 50 and 500 nM. RNA was extracted from the cells and then reverse transcribed to cDNA to measure the expression of (a) MMP-1, (b) MMP-13 and (c) TIMP-1 by Real-Time PCR as previously described. The results shown are expressed by relative quantification and normalised to 18s RNA (mean ± SD). Statistical significance was assessed where *P<0.05, **P<0.005 , ***P<0.001 using the bonferroni post-hoc test.

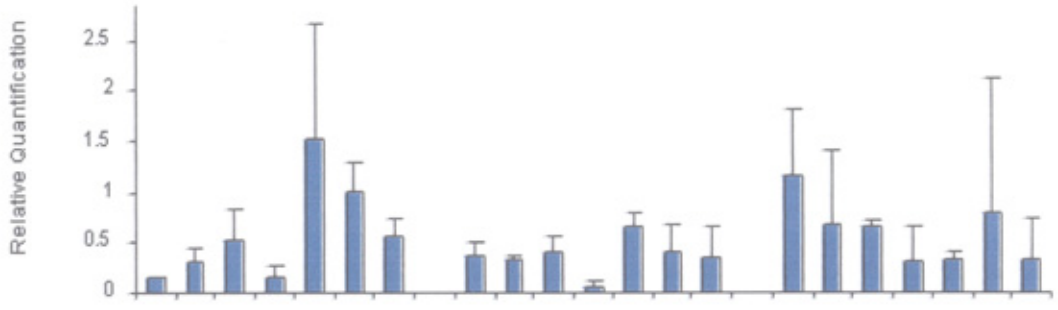
a) MMP-1



b) MMP-13



c) TIMP-1



IL-1α (ng/ml)	-	-	-	-	-	-	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
OSM (ng/ml)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	10	10	10	10	10	10			
IND (μM)	-	2	10	50	-	-	-	-	2	10	50	-	-	-	-	2	10	50	-	-	-			
INDHE (nM)	-	-	-	-	5	50	500	-	-	-	-	5	50	500	-	-	-	-	5	50	500			

The cDNA prepared as described in section 3.4.4.1 was analysed for the expression MMP-13 by Real-Time PCR. Results are shown as relative quantification (RQ) after being normalised to 18s (figure 3.8b). Relative quantification levels for MMP-13 in the control cDNA sample was 0.2 ± 0.01 RQ, in IL-1α cDNA the level was 0.45 ± 0.10 RQ, and in IL-1α + OSM cDNA the level was 2.0 ± 0.78 RQ. When indomethacin was add to the cells on its own no change was seen in the relative quantification level of MMP-13 gene expression, however when indomethacin heptyl ester was added at a dose of 5 nM there was a significant up regulation in the level of MMP-13 gene expression. Indomethacin heptyl ester at concentrations of 50 and 500 nM had no effect. The addition of indomethacin to IL-1α had no effect on the level of MMP-13 gene expression. The addition of the indomethacin heptyl ester at a concentration of 5 nM to IL-1α caused an increase in MMP-13 expression to 14.0 ± 2.0 RQ, while dose of 50 and 500 nM had no effect on gene expression. Indomethacin and indomethacin heptyl ester had no effect on the level of MMP-13 gene expression when added to IL-1α + OSM in combination. As indomethacin heptyl ester at 5 nM is well below the IC_{50} values for both COX-1 and COX-2 it may be possible that it is having an effect on an IL-1α signalling pathway as previously noted when investigating the effect on MMP-1 levels

3.4.4.3 The effect of indomethacin and indomethacin heptyl ester on TIMP-1 gene expression levels in human articular chondrocytes stimulated with IL-1α, and IL-1α + OSM

The cDNA prepared as described in section 3.4.4.1 was analysed for the expression TIMP-1 by Real-Time PCR. Results are shown as relative quantification (RQ) after being normalised to 18s (figure 3.8c). The relative levels for TIMP-1 in the control cDNA sample was 0.15 ± 0.02RQ; after stimulation with IL-1α the cDNA RQ level was 0.38 ± 0.14 RQ, and after stimulation with IL-1α + OSM the cDNA RQ level was 1.17 ± 0.64 RQ. However, the addition of indomethacin and indomethacin heptyl ester to cells without stimulation and after stimulation with IL-1 and IL-1 + OSM had no effect on levels of expression.

3.4.5 The effect of indomethacin and indomethacin heptyl ester on the synthesis of MMP-2 and -9 in human articular chondrocytes stimulated with IL-1α, and IL-1α + OSM

Figure 3.9 Effect of Indomethacin and indomethacin heptyl ester on MMP-2 and -9 levels in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.2ng/ml) and IL-1 (0.2 ng/ml) + OSM (10 ng/ml) in combination for 24 hours in the presence of indomethacin at 2, 10 and 50mM and indomethacin heptyl ester at 5, 50 and 500 nM. The media was harvested at 24 hours and assayed for MMP-2 and -9 by gelatin zymography as previously described.

To determine the effect of indomethacin and its COX-2 specific derivative indomethacin heptyl ester on the synthesis of MMP-2 and -9, both indomethacin and its derivative were added to human articular chondrocytes (HACs), which had been stimulated to resorb with cytokines IL-1α and IL-1 + OSM as described in section 3.4.3. Gelatinase activity levels were measured in harvested conditioned media by gelatin zymography.

3.4.5.1 The effect of indomethacin and indomethacin heptyl ester on the levels of MMP-2 and MMP-9 in IL-1α, and IL-1α + OSM stimulated human articular chondrocyte cultured medium.

Human articular chondrocytes digested from donated cartilage from knee replacement operations were allowed to grow to 80% confluence. The cells were serum starved overnight and were then stimulated with and without IL-1α, and IL-1α + OSM ± indomethacin or indomethacin heptyl ester for 24hr. The conditioned media was harvested and assayed for both MMP-2 and MMP-9 activity by gelatin zymography. In the control media a small amount of proMMP-2 and both pro and active MMP-9 were detected, whilst with IL-1α and IL-1 + OSM both pro and active MMP-2 and MMP-9 were increased. The addition of indomethacin to IL-1α treated chondrocytes caused a decrease in MMP-2 levels in a dose dependant manner, at 50 μM the levels of both the pro and active forms of MMP-2 and MMP-9 were moderately reduced, whilst only the levels of active MMP-2 were reduced with 2 and 10 μM and no effect was seen on MMP-9 levels. The addition of indomethacin heptyl ester to IL-1α and IL-1α + OSM had no effect on either MMP-2 or MMP-9 levels in the cultured medium.

3.5 DISCUSSION

In this chapter, the effect of indomethacin on the process leading to cartilage breakdown was investigated using both bovine cartilage explant cultures and human articular chondrocyte monolayers in culture. It is well documented that the addition of IL-1α + OSM to bovine nasal cartilage explants causes a reproducible up-regulation of the synthesis of collagenases, the rapid breakdown of the proteoglycan matrix, and almost complete degradation of total collagen by day 14 of culture (Cawston et al, 1995). The effect of stimulating human articular chondrocytes with IL-1α and OSM has also been

shown to cause the up-regulation of MMP-1, -2, -9, and -13 as well as an increase in TIMP-1 levels (Cawston et al, 1999).

The non-steroidal anti-inflammatory drug indomethacin is given to patients who are suffering from rheumatic diseases, with a standard dose regime of between 50 – 200 mg daily depending on the patient and disease severity leading to serum levels of 2.192 μg/ml. Indomethacin has also been shown to effectively inhibit the expression of MMPs and TIMPs in various cell types, but little or no evidence has been seen on its effect in the actual degradative process of cartilage breakdown. There is also very little known about the effect of a derivative of indomethacin, indomethacin heptyl ester specifically on its effects on proteoglycan, collagen, MMPs or TIMPs.

In this study, the stimulation of bovine cartilage explants and human articular chondrocytes with IL-1 + OSM was shown to cause the up-regulation of proteoglycan, collagen and collagenolytic activity from cartilage explants and induced MMP and TIMP expression in human articular chondrocytes, supporting previously published work.

As previously mentioned, few studies have investigated the effect of indomethacin and its derivative indomethacin heptyl ester on the actual process in the degradation of cartilage. However, the effect of indomethacin on proteoglycan release from cartilage has been widely debated with varying results being seen. A number of studies have shown that the release of proteoglycan from stimulated cartilage can in fact be inhibited by the addition of indomethacin (Dingle et al, 1999; McKenzie et, al 1976) whilst other studies have shown indomethacin to have no effect on proteoglycan release from stimulated cartilage (Jeffery and Aspden, 2007), data obtained in this study strongly agrees with the later, with neither indomethacin nor indomethacin heptyl ester being found to inhibit proteoglycan release. Therefore it is likely that the cleavage of the interglobular domain of aggrecan by proteinases such as aggrecanase is not inhibited by indomethacin or indomethacin heptyl ester. However the concentration of cytokines used was high and it may be a case that by reducing the cytokine concentration these agents may block the release of proteoglycan fragments, this may well be a factor as in the experiments carried out by McKenzie et al where inhibition was seen the concentrations of indomethacin used ranged from 5 – 20 μM, which were lower than those concentrations used in these experiments. The data in this study did however suggest that indomethacin is capable of blocking the release of collagen from the cartilage explants with indomethacin at 10 μM and 50 μM but not indomethacin heptyl ester, significantly down regulating collagen release, along with the

activity of collagenases. These effects have not previously been described in the literature. The concentrations of indomethacin where inhibition was seen are well above the IC_{50} values for COX -1 (740 nM) and COX-2 (970 nM) thus suggesting that by blocking COX-1 and COX-2, indomethacin is able to block collagen release from cartilage explants. However as these concentration are indeed far higher than the IC_{50} values the possibility that indomethacin may in fact be blocking the breakdown of cartilage by another mechanism entirely must be considered. The failure of indomethacin heptyl ester to affect the release of collagen is indeed interesting, especially considering its believed specificity for COX-2. However the difference in the structures of the two drugs may change their ability to infiltrate the cartilage matrix and therefore affect the assay system itself, with further investigation being required to investigate these findings.

The addition of indomethacin and indomethacin heptyl ester to stimulated human articular chondrocytes again showed some interesting results. At a protein level indomethacin was seen to inhibit the expression of both MMP-1 and -13 from IL-1α + OSM stimulated cells supporting previously published data (Takahashi et al, 1991) whilst with IL-1α only MMP-1 was seen to be inhibited by indomethacin. Interestingly the addition of indomethacin heptyl ester at 5 nM caused a significant up-regulation of both MMP-1 and MMP-13 in IL-1α and IL-1α + OSM stimulated HACs. This suggests that this dose of indomethacin heptyl ester may be in some way acting as an agonist and having a synergistic effect on IL-1 action, activating a pathway which results in the induction of MMP-1 and MMP-13, such as the mitogen-activated protein kinase pathway or nuclear factor-κB. When looking at the effect of indomethacin on TIMP-1 levels, it can be clearly seen that indomethacin is able to significantly inhibit the release of TIMP-1 from the stimulated HACs, this again supports data previously published by Krane et al, 1990. Indomethacin heptyl ester did not appear to affect TIMP-1 levels. Data showing the effect of indomethacin and its derivative at a gene level showed results very different to those seen at a protein level. Indomethacin did not appear to have any effect on MMP-1, MMP-13 or TIMP-1 expression by the cells, suggesting that indomethacin is blocking MMP-1 and MMP-13 at a translational level rather than through transcription. Indomethacin heptyl ester at COX-2 specific concentrations also had no effect of the expression of MMP-1, MMP-13 or TIMP-1. However, as seen at a protein level with the ELISA, at a dose below the COX-2 IC_{50} value indomethacin heptyl ester showed a clear and significant increase in the expression of MMP1 and MMP-13 in cells stimulated with IL-1α and IL-1α + OSM in the presence of

indomethacin heptyl ester alone. The effect on IL-1α and IL-1α + OSM supports the previous theory that indomethacin heptyl ester at a low dose may cause the activation IL-1α signalling pathway such as NF-κB by acting as an agonist to IL-1 and therefore causes super induction of MMP-1 and MMP-13.

Gelatin zymography data for the measurement of pro and active MMP-2 and MMP-9 showed that with both IL-1α, and IL-1α and OSM in combination, both pro and active MMP-2 and MMP-9 levels were increased. The addition of indomethacin to IL-1α stimulated cells caused a decrease in pro and active MMP-2 and MMP-9 levels with a dose of 50 μM, whilst lower doses had no effect. The addition of indomethacin heptyl ester had no effect on the synthesis of either of the gelatinases. This effect was again seen in the cells stimulated with IL-1α and OSM. This data for MMP-9 also supports previously published data by Ito et al, who found that indomethacin suppresses the production of MMP-9 in rabbit articular chondrocytes.

Whilst the differences in the results for each drug in this chapter are certainly interesting they are however somewhat unsurprising, as non-selective non-steroidal anti-inflammatory drugs are known to have differing effects on cartilage metabolism, with some being known to stimulate, some have no effect and others inhibit cartilage breakdown, with the reason behind this being largely unknown (Dingle et al, 1999; McKenzie et, al 1976; Mastbergen et al, 2002) The findings in this chapter suggest that further investigation is required to clearly understand the differences in the effects between indomethacin and indomethacin heptyl ester, with the differences in their structures alone being a key line of investigation.

Summary

Results in this chapter have shown that:

- Indomethacin and indomethacin heptyl ester have no effect on proteoglycan release from bovine nasal cartilage explants at the concentration of cytokines and reagents used.
- Indomethacin is able to suppress both collagen release and levels of collagenases released from bovine nasal cartilage explants.
- Indomethacin heptyl ester caused a small increase in collagen release but had no effect on the release of collagenolytic activity from bovine nasal cartilage.
- Indomethacin heptyl ester at 5 nM caused a major increase in the expression MMP-1 and MMP-13 both at a gene and protein level.

- Indomethacin inhibited MMP-2 and -9 levels in IL-1α, and IL-1α and OSM stimulated HACs, however its derivative had no effect.

CHAPTER 4: Effect of Statins on cytokine induced cartilage degradation

4.1 INTRODUCTION

Irreversible progressive loss of the cartilage matrix is a major feature in both rheumatoid arthritis (RA) and osteoarthritis which ultimately leads to the loss of joint function. Cartilage covers the articulating joint surface allowing free movement and is composed predominately of an extracellular matrix (ECM) containing proteoglycan and collagen. Embedded within this ECM are chondrocytes that regulate the turnover and remodelling of the cartilage matrix (Muir et al, 1995). Chondrocytes are recognised as important in the destruction of cartilage (Jubb et al, 1980). Proinflammatory cytokines such as interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα) are key molecules involved in the breakdown of normal cartilage. This cytokine stimulation causes a rapid loss of proteoglycan (GAG) (Saklatvala et al, 1984) which can be rapidly replaced, whilst the release of collagen is less rapid and is not easily replaced and so represents an irreversible step in cartilage degradation,

Matrix metalloproteinases (MMPs) are a family of enzymes involved in the cleavage of the extra-cellular matrix, in particular collagen, and can be detected in the sera, synovial fluids, synovial tissue and cartilage from patients with RA and OA (Yoshihara et al, 2000) The regulation of MMP activity is tightly controlled at various levels including; synthesis, pro-enzyme activation and inhibition of activated enzymes by tissue inhibitors of metalloproteinases (TIMPs) (Milner et al, 2003). The production of both MMPs and TIMPs is mediated by a variety of cytokines and growth factors including IL-1, OSM and TNFα, cytokines implicated in both RA and OA (Cawston et al, 1998).

The prevention of joint destruction is a key therapeutic target in both RA and OA. Anti-cytokine therapies are effective in RA but are expensive and not all patients respond (Keystone et al, 2005). There is an urgent requirement for small molecule therapies that target pathways involved in joint destruction as current OA treatments aim to reduce pain rather than prevent joint destruction.

The statins are a family of chemically related molecules that are used as cholesterol lowering drugs and are used extensively in medical practice and have been shown to reduce cardiovascular morbidity and mortality (Palmer et al, 2004). Recent studies have suggested that there are potential beneficial effects for using statins for the treatment of RA

(McCarey et al, 2004; Abeles et al, 2006). Statins are also thought to possess immunomodulatory properties and various studies have demonstrated that statins can prevent inflammatory arthritis in animal models (Wilson et al, 2005). More importantly a recent clinical trial demonstrated a significant improvement in disease severity and inflammation in RA patients (McCarey et al, 2004; Barsante et al, 2005) treated with statins. Statins have been studied in great detail in the cardiovascular field and been demonstrated to reduce MMP production (Luan et al, 2003; Wilson et al, 2005).

In this study we investigate if statins are able to block the breakdown of cartilage in a model system and whether or not the effect is dependant on the statin being hydrophilic or hydrophobic. We show for the first time that statins act in a chondroprotective manner by specifically blocking collagen release from cartilage stimulated to resorb with IL-1α and OSM, reducing the levels of collagenase activity and can regulate the levels of matrix degrading enzymes produced by human articular chondrocytes in response to pro-inflammatory stimuli.

4.2 AIMS

The aims of this chapter are to investigate the effect of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors pravastatin, mevastatin and simvastatin on cartilage degradation.

4.3 METHODS

IL-1α was diluted from stock solutions into culture medium and used at a final concentration of 1ng/ml in all experiments. Oncostatin M was diluted in culture medium to a final concentration of 10 ng/ml. All dilutions were prepared immediately prior to use and added to the plates on day 0 or day 7. Due to the hydrophobic nature of simvastatin and mevastatin, both drugs were made up to 1 mM stock concentrations in ethanol. Pravastatin was made up in sterile water to a stock concentration of 1 mM. All statins were further diluted in condition media to final assay concentrations.

4.3.1 Cell culture

Human articular chondrocytes were derived from articular cartilage obtained from consented patients following surgery. Enzymatic digestion of tissue and maintenance and culture of cells were as previously described in section 2.5. After the addition of test

reagents cells were incubated for 24 hours and the medium harvested for assay. All conditions were assayed in triplicate and primary chondrocytes were used at low passage (passage number 0).

4.3.2 Cartilage degradation assay

Bovine nasal septum cartilage was dissected and treated as previously described in section 2.2.1. The experiments were continued for 14 days. Bovine nasal cartilage discs were treated with mevastatin at a concentration of 20 μM, simvastatin at a concentration of 10 μM or pravastatin at a concentration of 10 μM with and without IL-1α and OSM. Culture supernates were harvested at day 7 and replenished with identical treatments to those on day 0, and the experiment was continued for a further seven days. Day 14 culture supernatants were harvested and the remaining cartilage was digested with papain.

4.3.3 Proteoglycan and collagen assay

Media samples and papain digests were assayed for sulphated glycosaminoglycans (as a measure of proteoglycan release) and hydroxyproline (as a measure of collagen degradation) as described in sections 2.2.2 and 2.2.3.

4.3.4 Enzyme assays

For experiments using human cells the collagenases MMP-1 and MMP-13 were assayed by specific ELISAs as described in section 2.4.

4.3.5 TaqMan Low density array

Extracted primary human articular chondrocytes were incubated with and without the combination of IL-1α and OSM either alone, or in the presence of simvastatin (10 μM) and pravastatin (10 μM). RNA was extracted from the cells, reverse transcribed and measured for gene expression by TaqMan low density array as describe in section 2.6.

4.4 RESULTS

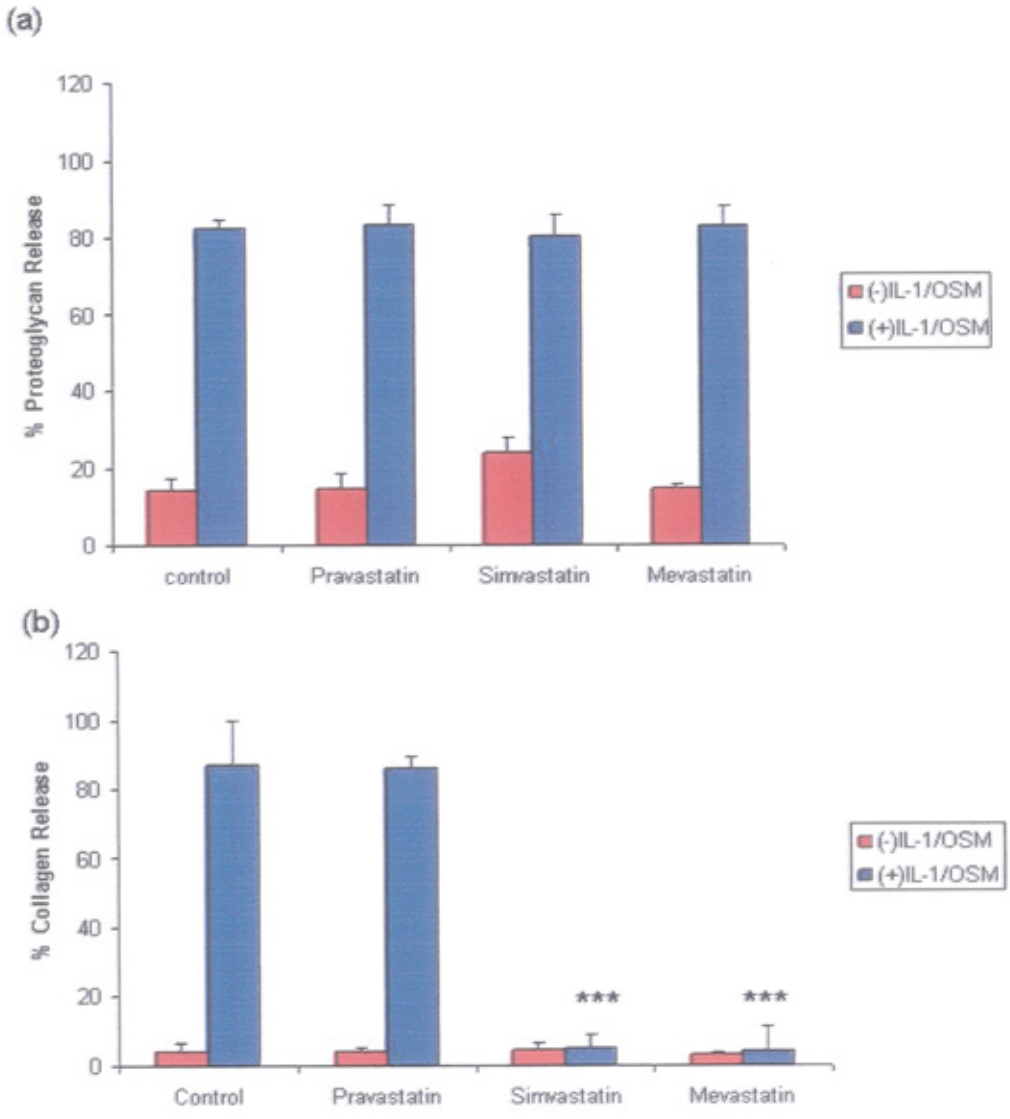
4.4.1 Effect of statins on the release of proteoglycan and collagen from bovine nasal cartilage treated with IL-1α and OSM.

Previous studies have shown that IL-1α in combination with OSM can reproducibly stimulate the release of both proteoglycan and collagen from bovine nasal cartilage in explant culture by day 7 and 14 respectively (Cawston et al, 1998). In this study, we use this combination as a cytokine stimulus to reproducibly promote cartilage degradation. Bovine nasal cartilage discs were cultured in control and IL-1α + OSM (1 + 10 ng/ml) containing media with and without mevastatin (20 μM), simvastatin (10 μM) and pravastatin (10 μM). In the presence of IL-1α + OSM approximately 82.6 % of cartilage proteoglycan was released in the day 7 media. Treatment with mevastatin, simvastatin and pravastatin resulted in no decrease in proteoglycan levels (figure 4.1a). After 14 days in culture with IL-1α + OSM, the level of collagen released into the media was found to be 87.0 ± 13.0 % compared to control levels of 4.3 ± 2.4 % (figure 4.1b). The addition of pravastatin did not have an effect on the release of collagen with levels 86.1 ± 3.3 %. Simvastatin and mevastatin significantly reduced collagen release to 5.4 ± 3.8 % and 4.4 ± 6.8 %.

4.4.2 The use of TaqMan low density arrays in the measurement of matrix metalloproteinase and tissue inhibitors of metalloproteinase gene expression in human articular chondrocytes (HAC) stimulated with IL-1α + OSM with and without pravastatin and simvastatin.

The measurement of the effect of pravastatin and simvastatin on the gene expression levels in IL-1α and OSM stimulated HACs was carried using the TaqMan low density array (TLDA). The stimulation of HACs with IL-1α + OSM caused an increase in the expression of MMP-1, -3, -8 and -13 of 0.54 ± 0.26, 18.25 ± 0.79, 0.02 ± 0.002 and 0.83 ± 0.24 respectively compared to control (figure 4.2a). The treatment of the IL-1α + OSM

Figure 4.1 Statins block the IL-1 and OSM induced collagen release but no proteoglycan release from bovine nasal cartilage.
Bovine nasal cartilage stimulated with IL-1 (1ng/ml) + OSM (10ng/ml) in the presence of mevastatin at 20μM, simvastatin 10μM and pravastatin 10μM for 7 and 14 days. The media was collected and replaced at day 7. Proteoglycan and collagen release was assayed *P<0.05, **P<0.005, ***P<0.001 using the Bonferroni post-hoc test.



stimulated chondrocytes with simvastatin (10μM) and pravastatin (10μM) gave varying results. With MMP-1 the addition of simvastatin and pravastatin resulted in a significant down regulation in expression of 0.19 ± 0.02 ($P \leq 0.01$) for simvastatin and 0.12 ± 0.004 ($P \leq 0.001$) for pravastatin. In the case of MMP-3, treatment with simvastatin and pravastatin resulted in MMP-3 being significantly up regulated by simvastatin to 27.5 ± 2.3 ($P \leq 0.01$) whilst pravastatin was able to suppress the expression of MMP-3 to 15.4 ± 0.86 ($P \leq 0.01$). With both MMP-8 and MMP-13, simvastatin and pravastatin were able to significantly inhibit the expression of these two collagenases to 0.008 ± 0.001 and 0.01 ± 0.001 for MMP-8 and 0.35 ± 0.04 and 0.42 ± 0.04 for MMP-13. In the case of the tissue inhibitors of metalloproteinases (TIMP), the stimulation of HACs with IL-1α and OSM caused an increase in the expression of TIMP-1 (1.54 ± 0.19) and TIMP-3 (0.17 ± 0.02) (figure 4.2). However, after treatment with simvastatin and pravastatin a variation in effects could be seen. In the case of TIMP-1 pravastatin decreased the expression to 1.13 ± 0.08, whilst simvastatin up regulated TIMP-1 expression to 1.75 ± 0.098. Expression of TIMP-3 was seen to be significantly down regulated with both pravastatin and simvastatin to 0.07 ± 0.008 and 0.08 ± 0.007 respectively.

4.4.3 Measurement of MMP-1 and MMP-13 in conditioned media from human articular chondrocytes (HAC) following stimulation with IL-1α + OSM with and without pravastatin, simvastatin and mevastatin.

To determine if these statins could block the production of either MMP-1 or MMP-13 from human articular chondrocytes (HAC) in culture the cells were treated with IL-1α + OSM in the presence and absence of pravastatin (10 μM), simvastatin (10 μM) and mevastatin (20 μM). Levels of MMP-1 were markedly elevated after stimulation with IL-1α + OSM of 279.5 ± 15.3 ng/ml (figure 4.3a), the addition of the statins showed varying effects. Pravastatin and simvastatin were seen to significantly reduce the expression of MMP-1 to 181.4 ± 51.4 ng/ml ($P =< 0.05$) and 167.9 ± 10.5 ng/ml ($P =< 0.001$) respectively.

The addition of mevastatin however did not result in the inhibition of MMP-1. Measurement of MMP-13 showed that like MMP-1 the levels were increased after treatment with IL-1α + OSM of 21.3 ± 1.0 ng/ml. However the effects seen after the addition of statins were distinctly different from those seen with MMP-1. The addition of simvastatin and mevastatin to IL-1α + OSM stimulated HACs caused a significant

Figure 4.2 The effect of statins on gene expression in IL-1 and OSM stimulated human articular chondrocytes.

Human articular chondrocytes were stimulated with IL-1 (0.02 ng/ml) and OSM (10 ng/ml) in the presence of Simvastatin 10 μM and Pravastatin for 24 hours. The expression of (a) MMPs and (b) TIMPs by the cells was assayed. *P<0.05, **P<0.005, ***P<0.001 using the Bonferroni post-hoc test.

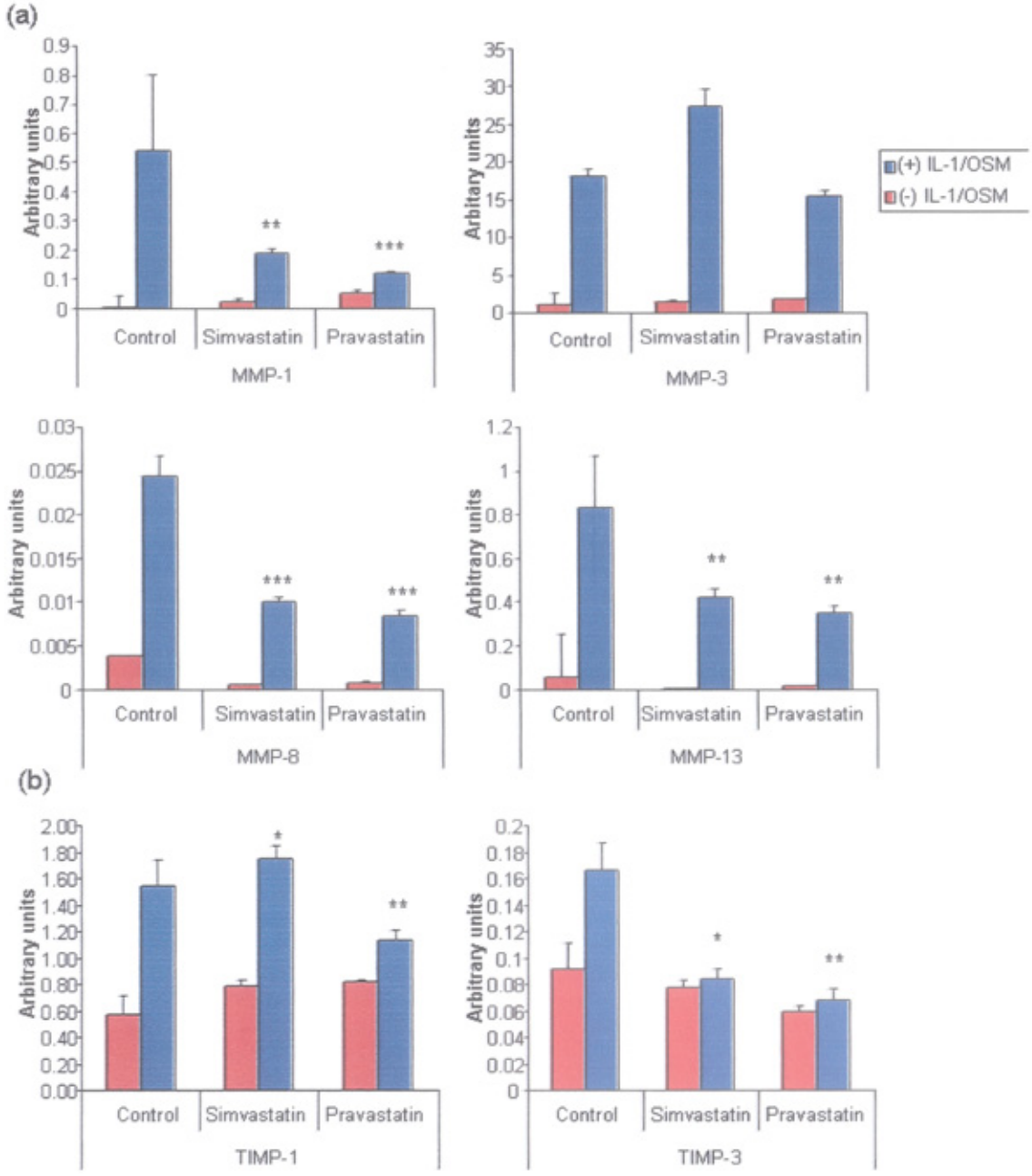
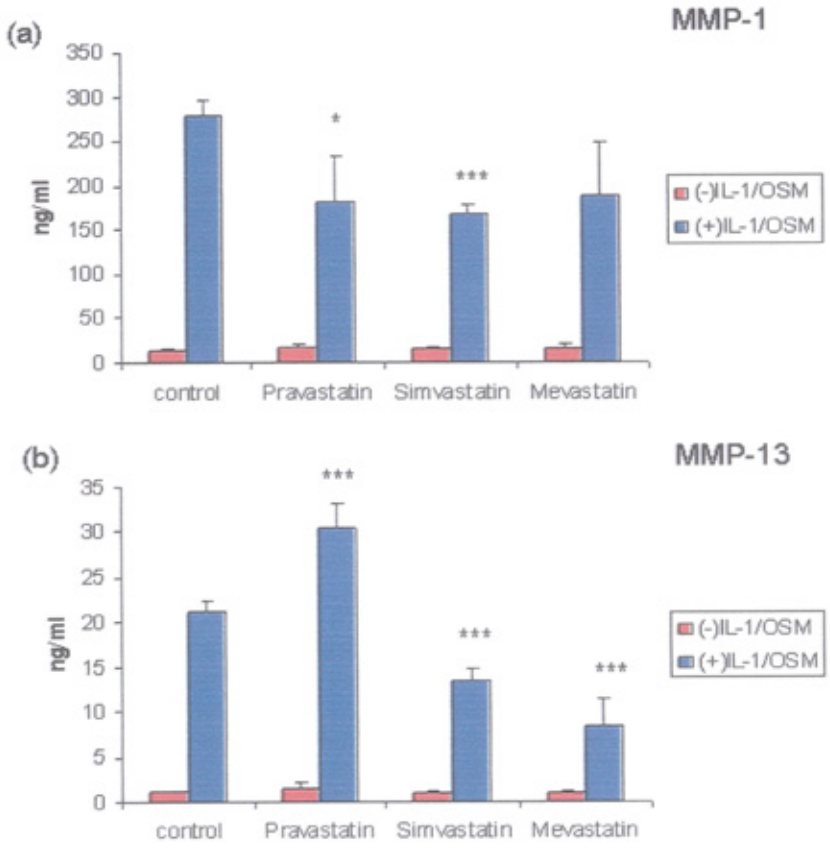


Figure 4.3 The effect of Statins on MMP-1 and MMP-13 levels in human articular chondrocytes.

Human articular chondrocytes were stimulated with IL-1 (0.2ng/ml) and OSM (2ng/ml) for 24 hours in the presence of Mevastatin at 20μM, Simvastatin 10μM and Pravastatin 10μM. The media was harvested after 24 hours and assayed for (a) MMP-1 and (b) MMP-13 by ELISA. **P*<0.05, ***P*<0.005, ****P*<0.001



inhibition of MMP-13 protein expression to 13.5 ± 1.3 ng/ml (P=<0.001) and 8.5 ± 3.0 ng/ml (P=<0.001) respectively (figure 4.3b). However, treatment with pravastatin resulted in a significant increase in the protein expression of MMP-13 of 30.4 ± 2.8 ng/ml (P=<0.001).

4.5 DISCUSSION

Statins are drugs which lower circulating cholesterol by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, one of the enzymes in the cholesterol biosynthesis pathway. In recent years they have also been shown to be of benefit in the treatment of cancer (Graaf et al, 2004) and diseases which involve inflammation including rheumatoid arthritis (Abeles et al, 2006; Barsante et al, 2005). Melavonate the product of HMG CoA reductase is not only used to make cholesterol, but is also used to synthesise dolicol phosphate, the lipid responsible for the transfer of N-linked glycans to proteins in the endoplasmic reticulum, and for the production of farnesyl and geranylgeranyl lipids which are transferred to proteins in the process of prenylation. It is this later step of protein prenylation which has been linked to its efficacy in cancer treatment as protein prenylation is crucial for the localisation of small G-protein signalling molecules such as Ras which are required to activate many down stream signalling pathways including the classical MAPK and NF-kB (Barsante et al, 2005; Graaf et al, 2004). Many of these signalling pathways have been implicated in the regulation of MMPs and arthritic disease.

Statins were investigated as a class of drugs which have been shown to have potential beneficial effects in treating RA and shown to be effective at preventing joint destruction in a collagen induced RA mouse model (Abeles et al, 2006). Atorvastatin has been demonstrated to be effective at preventing joint inflammation in a rat inflammatory RA model (Barsante et al, 2005) and perhaps more importantly a clinical trial has shown that this statin leads to a significant improvement in disease severity and inflammation in RA patients (Abeles et al, 2006). Protein prenylation has also been directly linked with arthritis as a farnesyltransferase inhibitor could significantly reduce the incidence and severity in a collagen induced arthritis mode (Na et al, 2004). The aim of this study was to determine if, in addition to these anti-inflammatory properties the statins could also act on pathways responsible for joint destruction.

In this study it has been shown for the first time that both mevastatin and simvastatin prevent the cytokine-initiated release of collagen from bovine nasal cartilage; however

pravastatin failed to have an effect on collagen release. No effect was seen with any of the statins on the release of proteoglycan from tissue. Treatment of human articular chondrocytes with pro inflammatory cytokines showed an up regulation of both MMP-1 and MMP-13 at a protein level, and MMP-1,-3,-8 and -13, and also TIMP-1 and –3 at a gene level.

At a protein level, pravastatin and simvastatin were both able to significantly block the release of MMP-1 from the IL-1α and OSM stimulated HACs, however mevastatin was unable to down regulate the expression of MMP-1. With MMP-13 both simvastatin and mevastatin were able to block the cytokine induced MMP-13 release; however pravastatin caused a significant up regulation. The use of the TaqMan low density array system allowed the ability of screening all or most MMPs, TIMPs and ADAMTS. Interestingly both simvastatin and pravastatin were able to prevent the augmentation of MMP-1, -8 and -13, and also TIMP-3. Simvastatin was seen to significantly up regulate the release of MMP-3 whilst pravastatin was able to block MMP-3 release, this effect was also seen with TIMP-1, where simvastatin increased TIMP-1 release but pravastatin caused a reduction.

This is the first report that sees statins block cytokine-induced breakdown in a bovine nasal explant culture and reduce the production of collagenases. What is clear is that there is a variation in the role that each statin is able to play, and this may be down to the statin being hydrophobic or hydrophilic. However, this direct effect of statins on chondrocytes and cartilage suggests that statins may potentially prevent cartilage breakdown in OA as well as RA. Statins are well suited as a treatment for OA as they are extremely well tolerated in patients over extensive periods of time and could also be used as a future treatment for OA. In conclusion, this study demonstrates the potential therapeutic role of statins in preventing cartilage damage in inflammatory joint diseases.

Summary

Results in this chapter have shown that:

- Statins have no effect on proteoglycan release from bovine nasal cartilage explants at the concentration of cytokines used.
- Simvastatin and mevastatin are able to suppress collagen release from bovine nasal cartilage explants.
- Simvastatin and pravastatin are able to down regulate the mRNA expression of collagenases MMP-1, -8 and -13.

- Simvastatin and pravastatin suppress mRNA expression of TIMP-3 whilst TIMP-1 is suppressed by pravastatin but up regulated by simvastatin.
- Statins pravastatin and simvastatin are able to suppress MMP-1 protein expression.
- Simvastatin and mevastatin inhibit MMP-13 protein expression, whilst it is up regulated by pravastatin.

CHAPTER 5: Sulfasalazine blocks the release of proteoglycan and collagen from cytokine stimulated cartilage and down regulates the matrix metalloproteinases.

5.1 INTRODUCTION

In both osteoarthritis (OA) and rheumatoid arthritis (RA), a progressive loss of cartilage matrix occurs ultimately leading to a loss of joint function. The cartilage matrix is made up of proteoglycan and collagen, and both are specifically cleaved during cartilage breakdown (Weyland and Goronzy, 1997). Matrix metalloproteinases (MMPs), a family of enzymes involved in extra-cellular matrix cleavage, can be detected in sera, synovial fluids, synovial tissue and cartilage from patients with OA and RA and are responsible for the cleavage of collagen (Yoshihara et al, 2000)

The proinflammatory cytokines interleukin-1α (IL-1α) and tumor necrosis factor α (TNFα) are key molecule's involved in promoting synovial proliferation and cartilage damage in disease, and are known to up regulate the production of MMPs (Mengshol et al, 2002). Oncostatin M (OSM) is a cytokine which is known to induce joint inflammation and cartilage damage in animal models (Cawston et al, 1998). Raised levels of OSM are detected in RA synovial fluid (Cawston et al, 1998) and these levels correlate with joint inflammation and the markers of collagen and aggrecan degradation in RA. OSM, in combination with IL-1α or TNFα, represents a potent and reproducible stimulus for the release of cartilage collagen by collagenolytic (Cawston et al, 1998) and gelatinolytic MMPs (Koshy et al, 2002).

Sulfasalazine (SSZ) is used widely in the treatment of inflammatory diseases such as rheumatoid arthritis and Crohn's disease (Rodenburg et al, 2000), and belongs to a class of drugs commonly known as disease modifying anti rheumatic drugs (DMARDs). It was produced in 1942 by combining the antibiotic sulfapyridine (SP) with an anti-inflammatory agent, 5-aminosalicylic acid (5-ASA). However the mechanisms of action of SSZ and its metabolites are not totally understood (Volin et al, 1999).

It is known that approximately 30% of SSZ is absorbed by the small intestine, whilst the remainder is broken down by colonic bacteria to its constitutive units of SP and 5-ASA prior to absorption (Volin et al, 1999).

Clinical and experimental evidence has shown that SSZ can affect a number of cellular functions that include cell proliferation (Aono et al, 1996), apoptosis (Oakley et al, 2005), proinflammatory cytokine production, MMP secretion (Hah and Lee, 2003) and prostaglandin E2 (PGE2) release (Nose et al, 1997). Many of these effects involve the nuclear factor κB (NF-κB) signalling pathway (Wahl et al, 1998). NF-κB is a multi subunit transcription factor that is a central mediator of the inflammatory response. In most cell types NF-κB is held as an inactive complex in the cytoplasm bound to its inhibitor IκB. Upon activation IκB is rapidly degraded and free NF-κB dimers translocate to the nucleus and activate the transcription of inflammatory cytokines, adhesion molecules and chemokines. Treatment of hepatocellular carcinoma cells with SSZ inhibits the activation of nuclear factor κB (NF-κB), down regulates inflammatory response genes and suppresses MMP-9 production (Hah and Lee, 2003). A study into inflammatory bowel disease showed that the mechanism of action of SSZ was to directly inhibit the IκB kinases IKK-α and IKK-β (Weber et al, 2000).

Treatment of synovial fibroblasts with SSZ effectively suppresses cell proliferation (Oakley et al, 2005), whilst also lowering the mRNA levels of MMPs and inflammatory cytokines (Oakley et al, 2005; Minghetti and Blackburn, 2000). When SSZ was added to normal IL-1β stimulated rabbit chondrocytes it suppressed glycosaminoglycan (GAG), collagen and prostaglandin E2 (PGE2) release, suggesting SSZ may have a protective effect on cartilage degradation (Nose et al, 1997).

In this study we have further investigated the possible chondroprotective nature of SSZ in a model cartilage system. We show, that SSZ acts in a chondroprotective manner by specifically blocking the release of glycosaminoglycan and collagen release from cartilage stimulated to resorb with IL-1α and OSM, reducing levels of collagenolytic activity and gelatinases, and by regulating the levels of MMP-1 and MMP-13 produced by human articular chondrocytes in response to pro-inflammatory stimuli.

5.2 AIMS

The aims of this chapter are to investigate the effect of sulfasalazine on cartilage degradation.

5.3 METHODS

Cytokines and growth factors were prepared as previously described in section 4.3. Sulfasalazine was diluted in DMSO to a stock concentration of 100 mM and diluted in condition media for assay concentrations.

5.3.1 Cell culture

Human articular chondrocytes were derived from articular cartilage obtained from consented patients following surgery. Enzymatic digestion of tissue and maintenance and culture of cells were as previously described in section 2.5.

5.3.2 Cartilage degradation assay

Bovine nasal septum cartilage was dissected and treated as previously described in section 2.2. The experiments were continued for 14 days. Bovine nasal cartilage discs were treated with sulfasalazine at concentrations of 1, 10, 100, 625 and 1000 μM with and without IL-1α and OSM. Culture supernatants were harvested at day 7 and replenished with identical treatments to those on day 0, and the experiment was continued for a further seven days. Day 14 culture supernatants were harvested and the remaining cartilage was digested with papain.

5.3.3 Proteoglycan and collagen assay

Media samples and papain digests were assayed for sulphated glycosaminoglycans (as a measure of proteoglycan release) and hydroxyproline (as a measure of collagen degradation) as described in sections 2.2.2 and 2.2.3.

5.3.4 Enzyme and Inhibitor assays

For experiments using human cells the collagenases MMP-1 and MMP-13 were assayed by specific ELISAs (Koshy et al, 2002). For experiments using bovine chondrocytes, collagenase activity was determined by diffuse fibril assay using ^{3}H-acetylated collagen. Amino phenyl mercuric acetate (APMA) was added at 0.7mM to activate pro collagenase. Inhibitory activity was assayed by the addition of samples to a known amount of active collagenase in the diffuse fibril assay. One unit of collagenase activity degrades 1μg of collagen per minute at 37^{o}C and one unit of inhibitory activity inhibits 2 units of collagenase by 50% as described in section 2.3.

5.3.5 Gelatin Zymography

Bovine cartilage conditioned media samples (day 14) were applied to gelatin containing SDS gels to measure MMP-2 and -9 level by zymography as described in section 2.10.

5.4 RESULTS

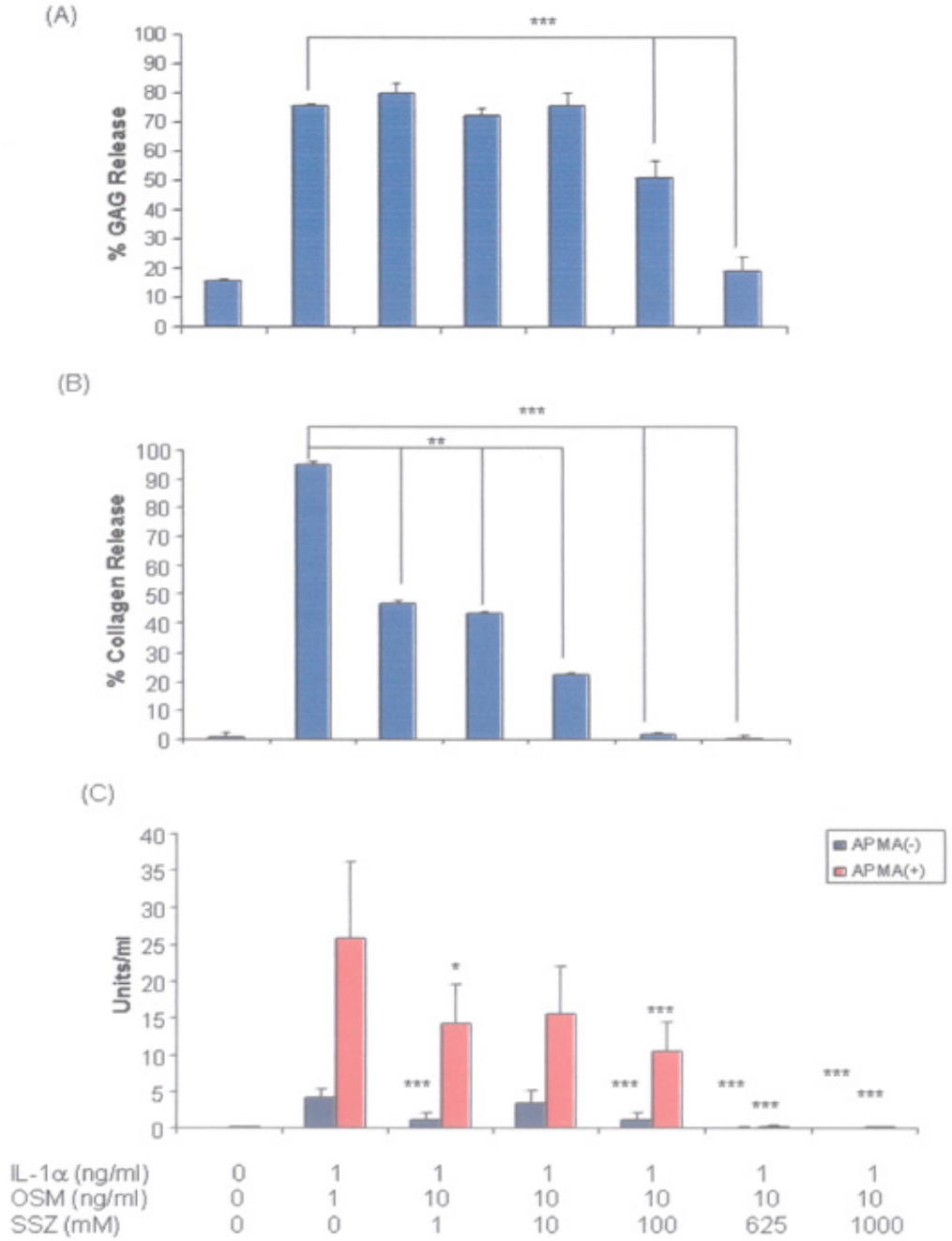
5.4.1 Effect of sulfasalazine on the release of glycosaminoglycan and collagen from bovine nasal cartilage treated with IL-1α and OSM.

Previous studies have shown the IL-1α in combination with OSM can reproducibly stimulate the release of glycosaminoglycans and collagen from bovine nasal cartilage in explant culture (Koshy et al, 2002). In this study we used this combination as the cytokine stimulus to reproducibly promote cartilage degradation and the release of cartilage glycosaminoglycans at day7 and collagen at day14 and investigated the effect of sulfasalazine in this in vitro system. Bovine nasal cartilage discs were cultured in control medium and IL-1α + OSM (1 + 10 ng/ml respectively) containing medium with and without sulfasalazine at 1, 10, 100, 625 and 1000 μM. In the presence of IL-1α and OSM approximately 75% of glycosaminoglycan was released to the medium at day 7, whilst approximately 95% cartilage collagen was released to the medium at day 14. In the presence of sulfasalazine a dose dependent reduction of glycosaminoglycan release was seen with significant inhibition at concentrations of 625 and 1000 μM sulfasalazine (figure 5.1a), whilst a dose dependent reduction of collagen release was seen with complete inhibition at concentrations of 625 and 1000 μM and partial, but significant inhibition at 1, 10 μM and 100 μM (figure 5.1b). Sulfasalazine alone had no effect on glycosaminoglycan and collagen release compared with control values and did not affect the release of LDH from the cartilage showing that, at the concentrations used, it was not toxic to the chondrocytes (data not shown).

5.4.2 The effect of sulfasalazine on the release of total and active collagenase activity in conditioned medium from bovine nasal cartilage.

Previous studies have shown that the increase of collagen release from bovine nasal cartilage is MMP dependant as it can be completely blocked by TIMP-1 or TIMP-2. The

Figure 5.1 Sulfasalazine blocks the IL-1 and OSM induced release of glycosaminoglycan , collagen and collagenolytic activity from bovine nasal cartilage. Bovine nasal cartilage was stimulated with IL-1 (1ng/ml) and OSM (10ng/ml) in the presence of sulfasalazine. The media was collected and replaced at day 7. **P*<0.05, ***P*<0.005 , ****P*<0.001 using the Bonferroni post-hoc test. (A) shows glycosaminoglycan release at day 7, (B + C) shows collagen release and collagenase activity at day 14.



conditioned medium from the bovine nasal cartilage stimulated with IL-1α and OSM with and without sulfasalazine was assayed in the presence and absence of amino phenyl mercuric acetate to measure both total and active collagenolytic activity respectively (figure 5.1c). IL-1α and OSM markedly stimulated the production of collagenase activity at Day 14 (both active and total) to a total of 25.7 ± 10.5 units per ml with 4.2 ± 1.0 units per ml in the active form. A dose dependent reduction was seen when cultures were treated with sulfasalazine from 1 to 1000μM. At the highest concentration of sulfasalazine (1000 μM) the levels of active collagenase were reduced to control levels whilst total collagenase levels were reduced to 0.3 ± 0.1 units per ml.

5.4.3 The effect of sulfasalazine on the release of MMP-2 and -9 in conditioned medium from bovine nasal cartilage.

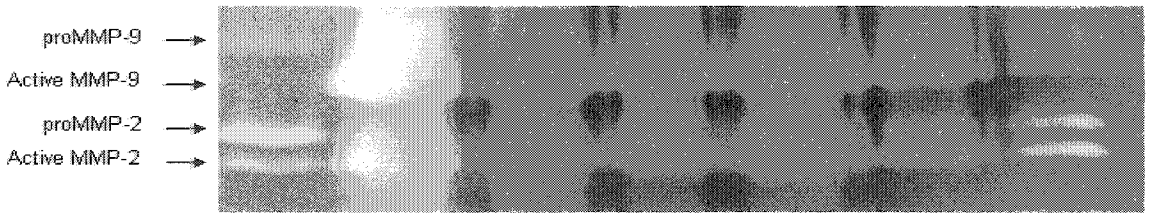
The conditioned medium from the bovine nasal cartilage stimulated with IL-1α and OSM with and without sulfasalazine was applied to a gelatin zymogram in order to determine if the levels of MMP-2 and MMP-9 were altered in the presence of sulfasalazine. IL-1α and OSM markedly stimulated the production of pro and active MMP-2 and -9 at Day 14 (figure 5.2). A dose dependent reduction was seen when cultures were treated with sulfasalazine from 1 to 1000 μM. At the highest concentration of sulfasalazine (1000 μM) the levels of pro and active MMP-9 were substantially reduced and pro and active MMP-2 was reduced to control levels.

5.4.4 Measurement of MMP-1 and MMP-13 in the conditioned media from human articular chondrocytes (HAC) following stimulation with IL-1α + OSM with and without sulfasalazine

To determine if sulfasalazine could block the production of either MMP-1 or MMP-13 from human articular chondrocytes (HAC) in culture these cells were treated with IL-1α + OSM in the presence and absence of sulfasalazine. Levels of MMP-1 and MMP-13 were markedly elevated after stimulation with IL-1α + OSM to 1629.6 ± 186.0 ng/ml (figure 5.3a) and 17.6 ± 4.3 ng/ml (figure 5.3b) respectively. The addition of sulfasalazine at concentrations 1 - 625 μM showed a significant inhibition of both MMP-1 and MMP-13 in the conditioned medium with sulfasalazine at 625 μM reducing levels to 168.5 ± 55.7 ng/ml for MMP-1 and 2.2 ± 0.3 ng/ml for MMP-13. The levels of MMP-1 and MMP-13 released by HACs stimulated by IL-1 alone at 0.02 ng/ml were also measured by ELISA.

Figure 5.2 Effect of sulfasalazine on the release of MMP-2 and MMP-9 from bovine nasal cartilage.

Bovine nasal cartilage was stimulated with IL-1 (1 ng/ml) and OSM (10 ng/ml) in the presence of sulfasalazine for 14 days. The media was collected and replaced at day 7. The release of MMP-2 and MMP-9 into the day 14 medium was assayed by gelatin zymography as described in the Methods and Materials. The results shown are for pro and active MMP-2 and MMP-9.



IL-1α (ng/ml)	0	1	1	1	1	1	1
OSM (ng/ml)	0	10	10	10	10	10	10
SSZ (μM)	0	0	1	10	100	625	1000

IL-1 increased MMP-1 levels to 968.3 ng/ml ± 184.4 ng/ml compared to control levels 140.9 ng/ml ± 52.5 ng/ml, sulfasalazine at concentrations of 10, 100 and 625 μM in the presence of IL-1 significantly reduced MMP-1 levels with 625 μM reducing MMP-1 to 204.4 ng/ml ± 32.9 ng/ml *($P \leq 0.001$)* (figure 5.3c). Sulfasalazine also reduced MMP-13 levels. IL-1α increased MMP-13 to 11.8 ng/ml ± 2.2 ng/ml compared to control 4.9 ng/ml ± 1.2 ng/ml, and was significantly inhibited by 1.9 ng/ml ± 0.3 ng/ml *($P \leq 0.001$)* in the presence of sulfasalazine at 625 μM (figure 5.3d), but not at lower doses of sulfasalazine

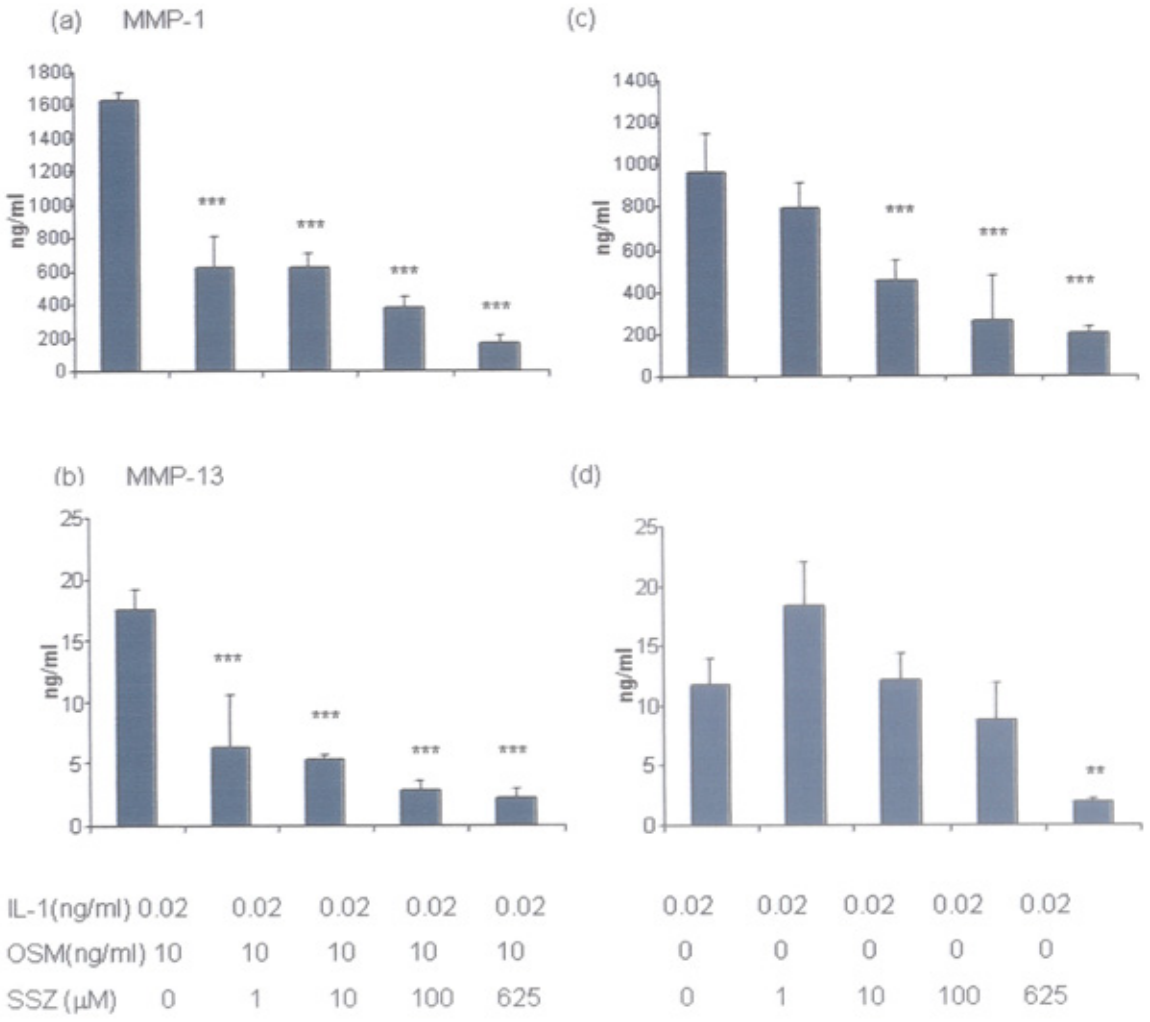
5.5 DISCUSSION

Disease modifying anti rheumatic drugs (DMARDs) are used in the treatment of RA. They are introduced at an early stage in the disease process and often on diagnosis as an initial aggressive form of treatment. Whilst there is no specific drug which is used first, in many cases sulfasalazine (SSZ) is often the initial drug of choice as it is effective with less side effects than methotrexate. At an oral dose of 3 g/day, the mean serum concentration of SSZ is 6.0 μg/ml (15.0 μM) with similar concentrations detected in synovial fluid (Aono et al, 1996). However, the mechanism of action of this DMARD is not completely understood and recent studies have investigated how sulfasalazine is effective. In a study using monocyte derived macrophages and macrophage cell-lines found that sulfasalazine inhibited TNF-α expression which may be due to the induction of apoptosis. TNF-α induced MMP-9 has been shown to be completely inhibited by sulfasalazine in hepatocellular carcinoma cells (Hah and Lee, 2003), whilst it has also been seen to inhibit basis fibroblastic growth factor induced chemotaxis, inhibiting cell proliferation and decreasing IL-8 expression in human dermal micro vascular endothelial cells (Aono et al, 1996).

One study looking at the effect of sulfasalazine on intracellular signalling pathways has suggested that sulfasalazine is a potent and specific inhibitor of NF-κB (Wahl et al, 1998). A further study suggests that the inhibition of NF-κB is effected by sulfasalazine directly suppressing IκB-α phosphorylation and its subsequent degradation, thus suggesting the ability of sulfasalazine to block IKK or an upstream signal (Weber et al, 2000).

Figure 5.3 Effect of Sulfasalazine on MMP-1 and MMP-13 levels in human articular chondrocyte conditioned media following stimulation with IL-1 and OSM.

Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) for 24 hours in the presence of sulfasalazine. The media was harvested after 24 hours and assayed for (A + C) MMP-1 and (B + D) MMP-13 by ELISA as previously described. *P<0.05, **P<0.005 , ***P<0.001 using the bonferroni post-hoc test.



In this study we investigated sulfasalazine, a drug known to be effective in the prevention of inflammation in rheumatoid arthritis, as a drug capable of having chondroprotective properties. Previous studies have shown modest and contradictory effects of sulfasalazine. It was found to dose dependently suppress the release of glycosaminoglycans, collagenase activity, MMP-3 and PGE_2 from rabbit chondrocytes treated with IL-1β (Nose et al, 1997). However another study, looking at steady state mRNA levels in RA synovial fibroblasts, showed that sulfasalazine suppressed the expression of tumor necrosis factor – alpha (TNF-α), MMP-3 and MMP-1 but elevated mRNA levels for TIMP-2 and MMP-2 (Minghetti and Blackburn, 2000). Sulfasalazine significantly inhibited RA synovial fibroblast proliferation and IL-1 and IL-6 production, whilst also inhibiting the over expression of c-fos, thus suggesting that sulfasalazine may inhibit synovial fibroblast proliferation without the involvement of other cellular factors and the authors suggest this may be the mechanism of clinical remission induced by this DMARD in RA patients (Oakley et al, 2005).

In this study, it is shown for the first time that sulfasalazine prevented IL-1α and OSM initiated glycosaminoglycan and collagen release from an in vitro bovine nasal cartilage model; this was accompanied by a concomitant decrease in active collagenase and total collagenase activity in the bovine nasal cartilage conditioned media. An inhibitory effect was also seen on both pro and active MMP-2 and -9. Consistent results were obtained when we investigated the effect of sulfasalazine in human articular chondrocytes. When stimulated with proinflammatory cytokines both MMP-1 and MMP-13 were up regulated. Sulfasalazine blocked the release of MMP-1 from IL-1α and IL-1α + OSM stimulated cells in a dose dependant manner. The effect of sulfasalazine on MMP-13 showed a dose dependant decrease in IL-1α + OSM stimulated cells, whilst inhibition was seen only with at the highest concentration of sulfasalazine in cells stimulated with IL-1α alone.

This is the first time that sulfasalazine has been shown to block cytokine-induced cartilage collagen breakdown in cartilage explant culture, whilst also reducing the production of the gelatinases MMP-2 and MMP-9. It is also the first example of sulfasalazine blocking MMP-1 and MMP-13 produced from cytokine stimulated human articular chondrocytes (HAC) in a monolayer culture. This effect by sulfasalazine upon chondrocytes and cartilage suggests that in addition to its anti-inflammatory properties it may also have a potential therapeutic role in preventing cartilage damage in both inflammatory joint disease such as rheumatoid arthritis and non-inflammatory disease such as osteoarthritis.

Summary

Results in this chapter have shown that:

- Sulfasalazine is able to suppress proteoglycan release, collagen release and collagenase activity from bovine nasal cartilage explants.
- Sulfasalazine inhibited MMP-2 and -9 synthesis in medium from IL-1α + OSM stimulated bovine nasal cartilage explants.
- Sulfasalazine suppressed MMP-1 and MMP-13 protein expression in IL-1α and IL-1α + OSM stimulated human articular chondrocytes.

CHAPTER 6: The effect of sulfasalazine on the gene expression levels of MMP, TIMP and ADAMTS in human articular chondrocytes.

6.2 INTRODUCTION

A number of studies have been carried out looking at gene expression levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) or ADAMTS (a disintergrin and metalloproteinase domain with thrombospondin motifs) in osteoarthritis cartilage and synovium, as well as in human articular chondrocytes in a monolayer culture. One particular study has looked at the difference in the gene expression of MMPs, TIMPs and ADAMTS between normal cartilage and cartilage from patients with OA. Their data showed a significant increase in the levels of MMP-2, -9, -13 and -16, and ADAMTS-2, -3, -12, and -14, whereas MMP-1, -3 and -10, and ADAMTS-1, -5 and -9 levels were shown to decrease in diseased cartilage compared to normal cartilage (Kevorkian et al , 2004). A similar study was carried out comparing the expression of MMPs, TIMPs and ADAMTS in normal cartilage to OA disease cartilage, and also the variations in gene expression in normal synovium and OA synovium. They found that MMP-9, -13 and -16, and ADAMTS-2 were up regulated in both the OA cartilage and synovium, whilst MMP-10 and ADAMTS-1, -4 and -5 were found to be down regulated in both tissues (Davidson et al, 2006). Interestingly, some of the genes, such as MMP-1 and -3, which were found to be downregulated in OA cartilage and synovium compared to normal cartilage, have been shown to increase in studies investigating RA synovium by histology (Tetlow et al, 1998), synovial fluids (Walakovits et al, 1992 and Clark et al, 1993) and sera (Green et al, 2003). In studies using monolayer cultures, where mRNA was extracted from primary human articular chondrocytes stimulated with IL-1β, they found that a high increase was seen in the levels of expression of MMP-1, -3 and -13 in chondrocytes from early stage OA, whilst MMP-3 levels were decreased in chondrocytes from late stage OA. ADAMTS-5 and MMP-14 were expressed in all groups, whilst ADAMTS-4 was induced by IL-1β in human articular chondrocytes (Bau et al, 2002). Another study using the human chondrocyte cell line T/C28a4, showed that when stimulated with IL-1α and OSM, an induction is seen in the expression of MMP-1, -3, -8, -13 and -14, as well as ADAMTS-4 (Koshy et al, 2002). These studies all show that specific members of the MMP, TIMP and ADAMTS subset of

families can be specifically up regulated or down regulated and this varies with the disease type and on the extent of the disease.

Sulfasalazine was developed by a Swedish physician called Dr Nana Swartz in the late 1930s and first reported in 1942. It was produced by coupling a salicylate known as 5-aminosalicylic acid and a sulfa antibiotic known as sulfapyridine together with an azo bond, which is then split by bacterial-azo-reductase in the large intestine. Whilst there are various studies looking at the gene expression in cartilage, there are very little or no data investigating the affect of sulfasalazine on mRNA expression in human articular chondrocytes, and especially its effects on the mRNA levels of MMPs, TIMPs or ADAMTS. However, two studies have been carried out looking into the effect of sulfasalazine on steady state mRNA levels in rheumatoid synovial fibroblasts. In one study, it has been shown that sulfasalazine is able to suppress the expression of MMP-3 after stimulation with IL-1α, whilst elevating the expression of MMP-1 and MMP-2 (Minghetti and Blackburn, 2000). Another study, looking at the effect of sulfasalazine on rheumatoid synovial fibroblasts, showed that it is able to inhibit the production of interleukin-1 beta (IL-1β) by synovial cells both at a protein and mRNA level. However, both of these studies looked at only a small number of metalloproteinases.

6.3 AIMS

The aims of this chapter are to investigate the affect of sulfasalazine on the gene expression of MMPs, TIMPs and ADAMTS in human articular chondrocytes stimulated with IL-1α and OSM in combination.

6.4 METHODS

6.4.1 TaqMan Low Density Arrays

Extracted primary human articular chondrocytes were incubated with and without the combination of IL-1α [0.2 ng/ml] and OSM [10 ng/ml) either alone, or in the presence of sulfasalazine [1 – 1000 μM]. RNA was extracted from the cells, reverse transcribed and measured for gene expression as described in section 2.6.

6.5 RESULTS

6.4.1 The effect of sulfasalazine on the relative expression levels of metalloproteinase genes involved in the processes of joint destruction.

In order to investigate the effect of sulfasalazine on the relative gene expression of genes know to be involved in the process of cartilage and joint destruction, sulfasalazine was added to human articular chondrocytes stimulated with IL-1α and OSM.

Human articular chondrocytes digested from donated hip or knee cartilage were grown to ~80% confluence. The cells were serum starved overnight and were then stimulated with and without IL-1α and OSM ± sulfasalazine for 24hr, after which the cells were lysed and RNA extracted using Qiagen RNeasy kits before being reverse transcribed into cDNA. The cDNA was run on a TaqMan low density array for analysis of MMPs, TIMPs and ADAMTS by Real-Time PCR after being normalised to house keeping gene 18s. In the experiment shown, the levels of expression for individual genes are demonstrated by use of a heat map (figure 6.1), which is intended to show variation in the cycle threshold (cT) value for each gene. A colour scale is used to clearly present the expression levels, with genes which are very highly expressed indicated by the colour red and genes which are below detection being presented by the colour blue. The heat map clearly shows that genes known to be involved in the degradative process of cartilage breakdown such as MMP-1 and MMP-3 are highly expressed compare to genes which are not thought to be involved such as ADAMTS-18 and MMP-20.

6.4.2 The effect of sulfasalazine on the gene expression levels of the classical collagenases MMP-1, MMP-8 and MMP-13 in human articular chondrocytes.

In order to quantify the effect of sulfasalazine on the gene expression levels of MMP-1, -8 and -13 during cartilage breakdown, the average cT value results were taken from the heat map and $2^{-\Delta}C_T$ was calculated from this value giving an arbitrary unit value. Standardising across genes was carried out by plotting the results for each gene as a fold induction compared to the control value.

In the experiment shown, IL-1α and OSM caused an induction of MMP-1 release (10.8 ± 2.0) compared to control of 1.0 ± 0.2 (figure 6.2a). The addition of sulfasalazine at 625 and 1000 μM caused significant reduction in the up regulation by IL-1α and OSM to 2.4 ± 0.9 and 3.0 ± 1.0 respectively. These results support previous data seen in chapter 5,

Figure 6.1 The effect of sulfasalazine on relative gene expression levels for metalloproteinases and their inhibitors in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) in combination for 24 hours in the presence of sulfasalazine (SSZ) at 1, 10, 100, 625 and 1000 μM. RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measure by TaqMan low density array as previously described. Results shown are expressed as a CT value.

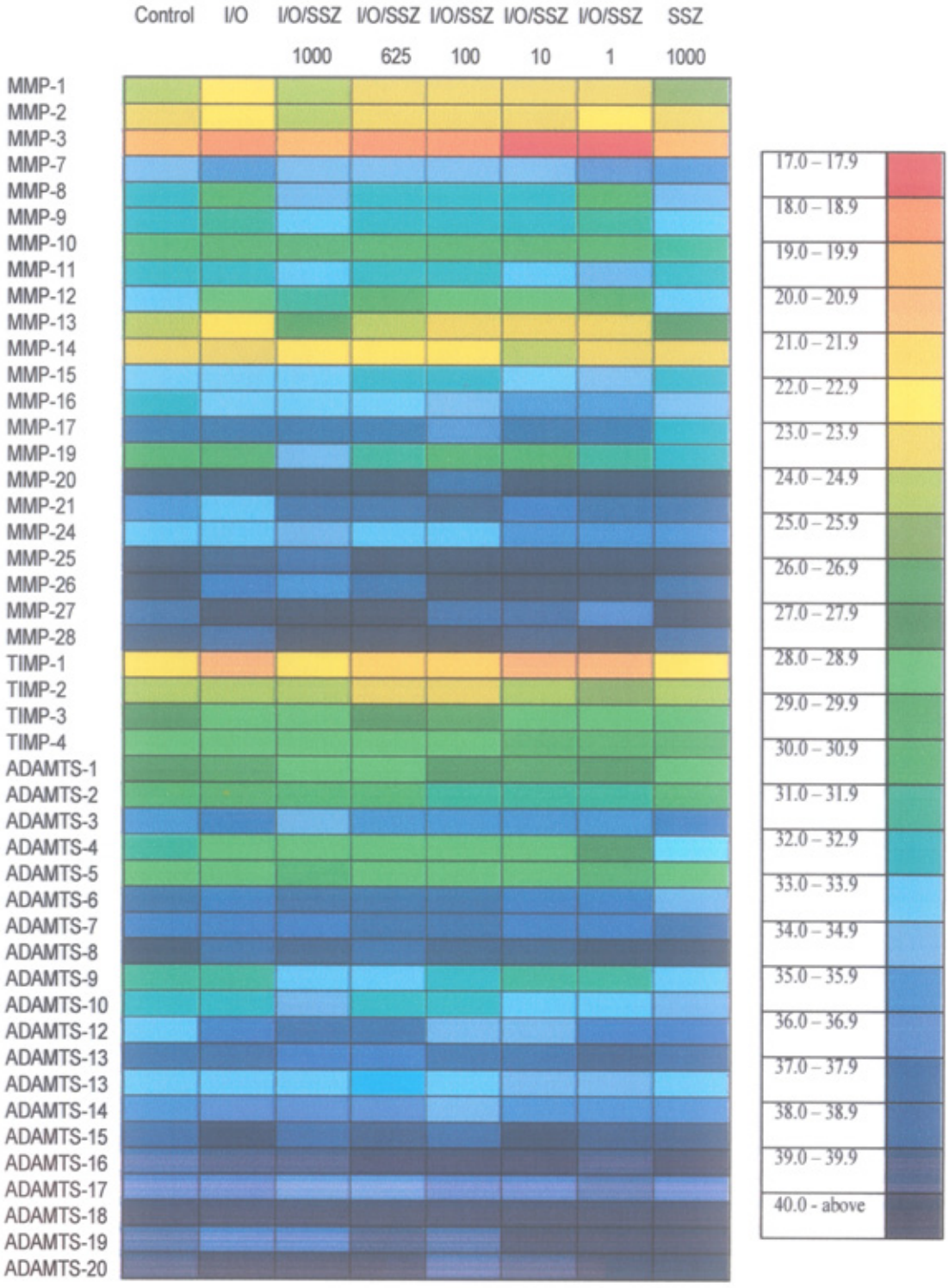
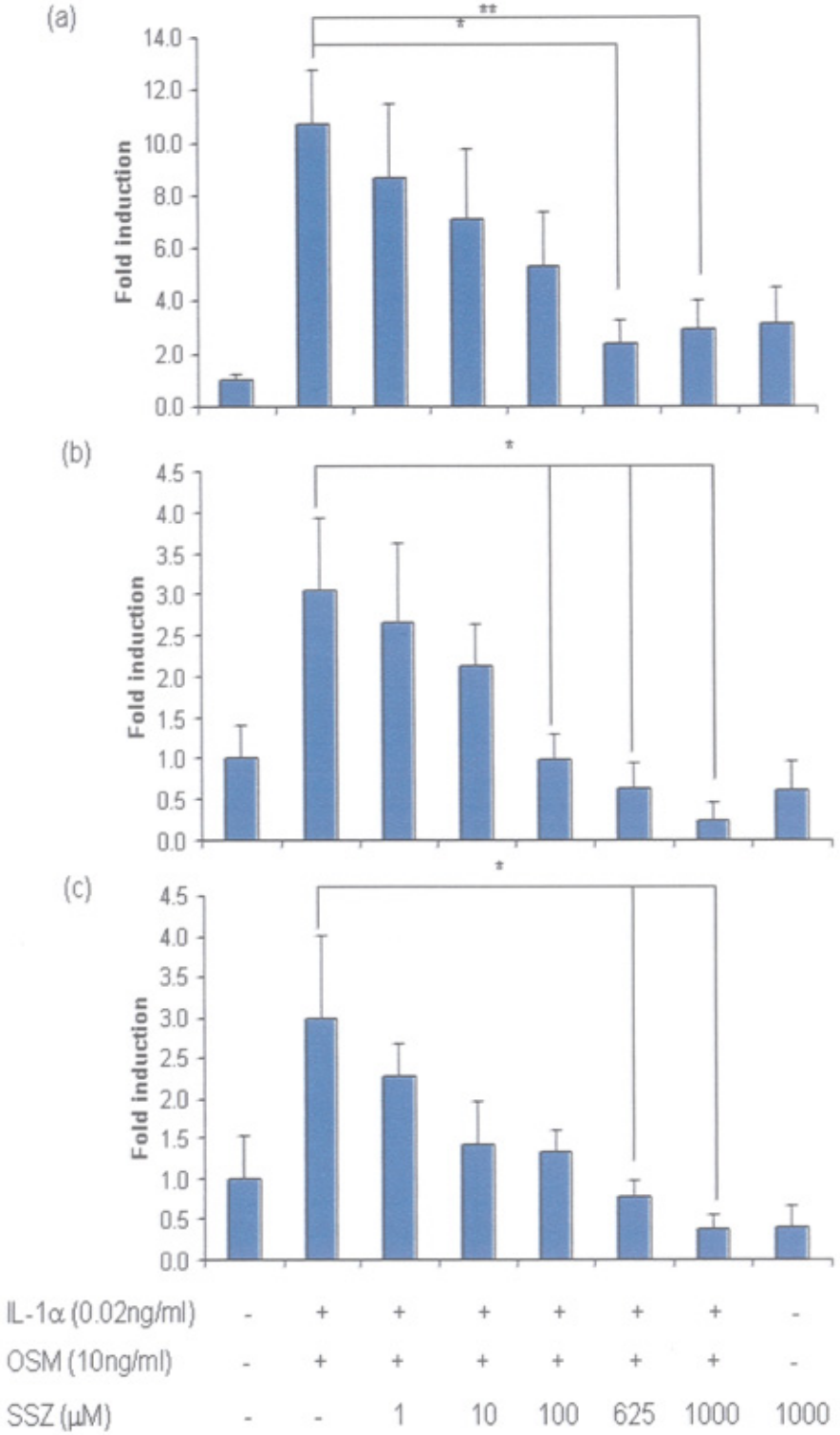


Figure 6.2 The effect of sulfasalazine on the gene expression levels of collagenases in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) in combination for 24 hours in the presence of sulfasalazine (SSZ) (1, 10, 100, 625 and 1000 μM). RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measured for (a) MMP-1, (b) MMP-8 and (c) MMP-13 by TaqMan low density array as previously described. Results shown are expressed as a fold induction of control (mean ± SD). Statistical significance was assessed where * $P<0.05$, ** $P<0.005$, *** $P<0.001$ using one-way ANOVA with bonferroni post-hoc test. (n=5)



where MMP-1 was significantly down regulated by sulfasalazine at a protein level in media harvested from human articular chondrocyte in monolayer.

The results obtained from the TaqMan low density array in this experiment showed an induction in the levels of MMP-8 expression by IL-1α and OSM of 3.0 ± 0.9 compared to the control levels of 1.0 ± 0.4 (figure 6.2b). The expression levels for MMP-8 for sulfasalazine in the presence of IL-1α and OSM showed that sulfasalazine at its three highest were able to significantly block the effect of IL-1α and OSM with MMP-8 expression levels being just 0.2 ± 0.2 with sulfasalazine at its highest concentration of 1000 μM.

The level of expression for MMP-13 was assessed from the microfluidic card data. Expression was seen to be induced by IL-1α and OSM to a level of 3.0 ± 1.0 compared the control levels of 1.0 ± 0.5 (figure 6.2c). The addition of sulfasalazine in the presence of IL-1α and OSM showed that the two highest concentrations of 625 and 1000 μM significantly inhibited MMP-13 expression to 0.8 ± 0.2 and 0.6 ± 0.2 respectively. This again supports data shown in chapter 5, where MMP-13 was downregulated by sulfasalazine at a protein level.

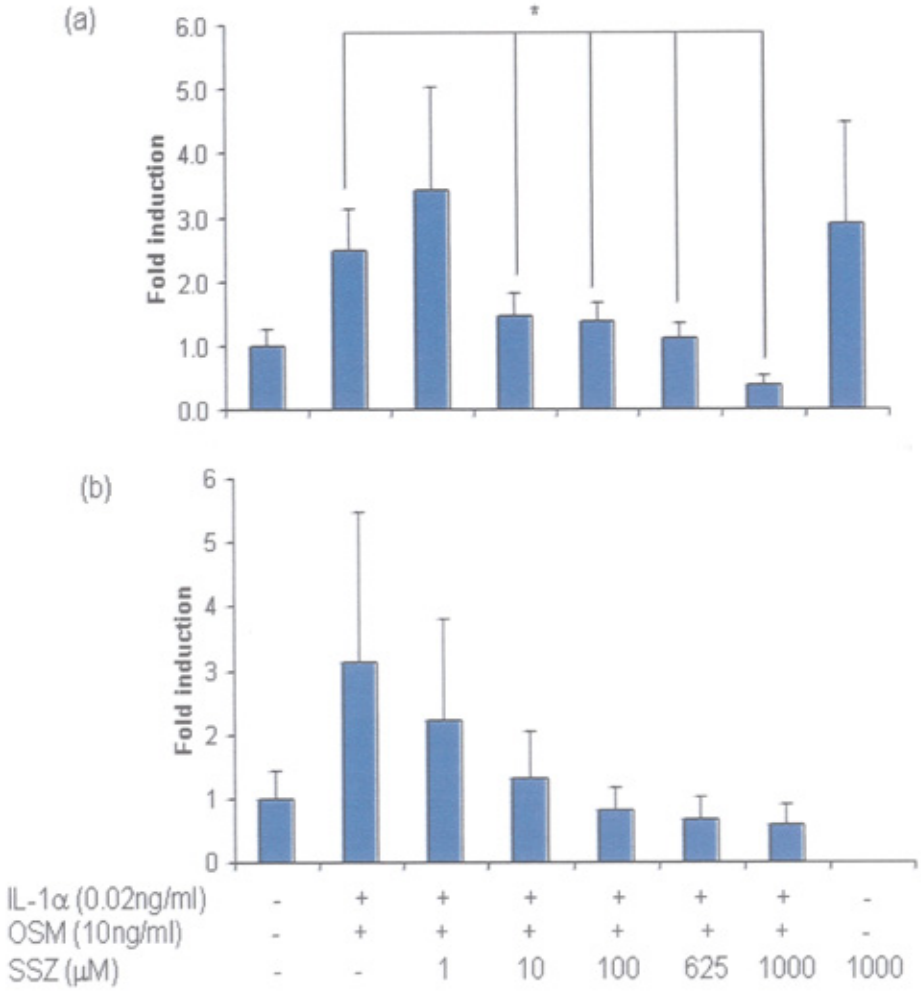
6.4.3 The effect of sulfasalazine on the gene expression levels of gelatinases MMP-2 and -9 in human articular chondrocytes.

In order to quantify the effect of sulfasalazine on gene expression levels of MMP-2 and -9 in human articular chondrocytes, their expression was detected by the addition of cDNA to the TaqMan low density arrays as in section 6.3.2.

The expression of MMP-2 is induced by the addition of IL-1α and OSM, where levels were increased to 2.5 ± 0.6 compared to control levels of 1.0 ± 0.3 (figure 6.3a). The addition of sulfasalazine to chondrocytes stimulated with IL-1α and OSM cause a reduction in the expression levels of MMP-2 with all concentrations tested above 1 μM, with 1000 μM reducing MMP-2 expression to 0.4 ± 0.2. Sulfasalazine therefore blocks the increase of MMP-2 expression caused by IL-1α and OSM.

In the experiment shown, IL-1α and OSM showed an up regulation in the expression of MMP-9 of 3.1 ± 2.4 compared that of control where MMP-9 expression was 1.0 ± 0.4 (figure 6.3b). The addition of sulfasalazine to the IL-1α and OSM caused no significant inhibitory effect on MMP-9.

Figure 6.3 The effect of sulfasalazine on the gene expression levels of gelatinases in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) in combination for 24 hours in the presence of sulfasalazine (SSZ) (1, 10, 100, 625 and 1000 μM). RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measured for (a) MMP-2 and (b) MMP-9 by TaqMan low density array as previously described. Results shown are expressed as a fold induction of control (mean ± SD). Statistical significance was assessed where * $P<0.05$, ** $P<0.005$, *** $P<0.001$ using one-way ANOVA with bonferroni post-hoc test. (n=5)



6.4.4 The effect of sulfasalazine on the gene expression levels of stromelysins MMP-3, -10 and -11 in human articular chondrocytes.

In order to quantify the effect of sulfasalazine on gene expression levels of the stromelysins MMP-3, -10 and -11 during cartilage breakdown, their expression was detected by the addition of cDNA to the TaqMan low density arrays as in section 6.3.2. The addition of IL-1α and OSM to the human articular chondrocytes caused an induction in the gene expression level of MMP-3 from a control level of 1.0 ± 0.3 to 3.1 ± 0.6 (Figure 6.4a). The inclusion of sulfasalazine to the IL-1α and OSM caused a significant inhibition in the levels of MMP-3 gene expression with sulfasalazine at 100, 625 and 1000 μM of 1.7 ± 0.4, 1.4 ± 0.3 and 1.1 ± 0.3 respectively.

The level of MMP-10 gene expression in unstimulated cells was found to be 1.0 ± 0.5 (figure 6.4b). After stimulation with IL-1α and OSM the expression of MMP-10 was up regulated to a level of 7.7 ± 5.7. The addition of sulfasalazine showed a dose responsive down regulation with the highest concentration reducing the level of MMP-10 to 2.3 ± 1.1. However, due to the IL-1α and OSM having a large error bar this was not significant.

The addition of IL-1α and OSM did not cause an induction in the expression level of MMP-11. The inclusion of sulfasalazine to IL-1α and OSM reduced the level of MMP-11, but this did not reach significance (figure 6.4c).

6.4.5 The effect of sulfasalazine on the gene expression levels of membrane type – metalloproteinases, MMP-14, -15 and -16 in human articular chondrocytes.

In order to quantify the effect of sulfasalazine on gene expression levels of the membrane type - metalloproteinases MMP-14, -15 and -16 in human articular chondrocytes, their expression was detected by the addition of cDNA to the TaqMan low density arrays as in section 6.3.2.

The addition of IL-1α and OSM resulted in no clear effect on the induction of MMP-14, -15 or -16 expression (figure 6.5). The addition of sulfasalazine showed no clear pattern in changing the expression levels and at no concentration was significance reached.

Figure 6.4 The effect of sulfasalazine on the gene expression levels of stromelysins in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) in combination for 24 hours in the presence of sulfasalazine (SSZ) (1, 10, 100, 625 and 1000 mM). RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measured for (a) MMP-3, (b) MMP-10 and (c) MMP-11 by TaqMan low density array as previously described. Results shown are expressed as a fold induction of control (mean ± SD). Statistical significance was assessed where * $P<0.05$, ** $P<0.005$, *** $P<0.001$ using one-way ANOVA with bonferroni post-hoc test. (n=5)

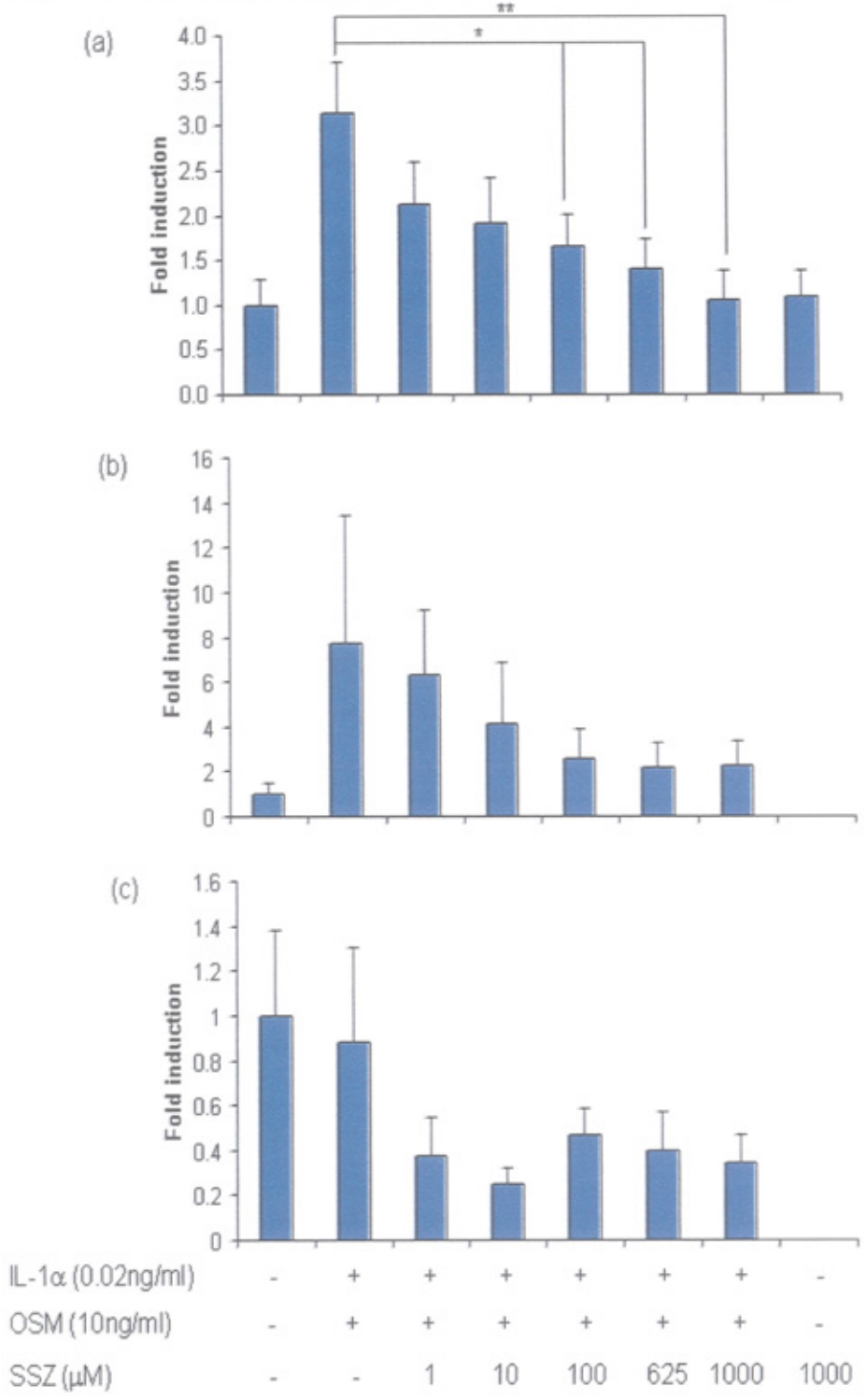
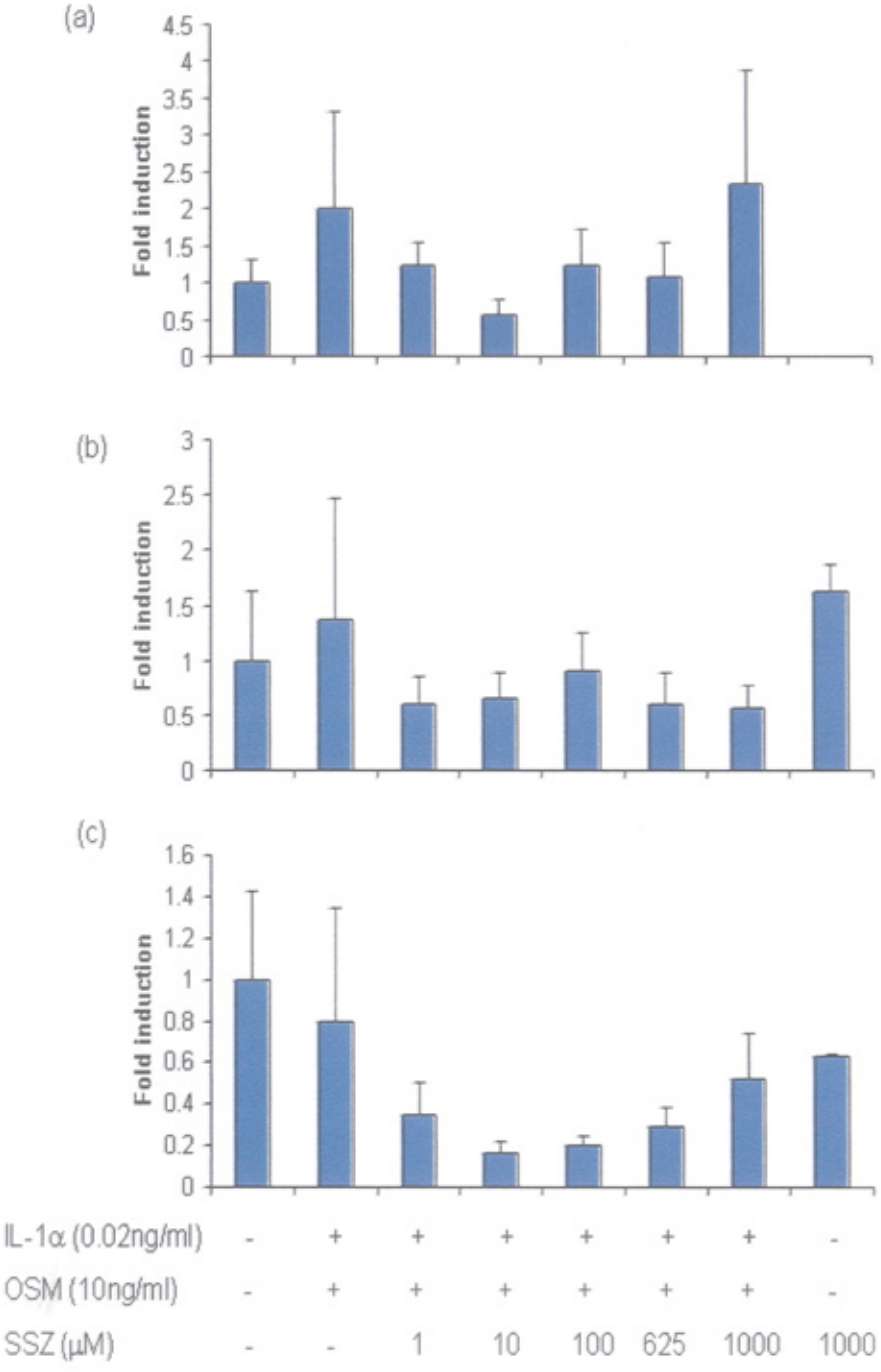


Figure 6.5 The effect of sulfasalazine on the gene expression levels of membrane type metalloproteinases in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) in combination for 24 hours in the presence of sulfasalazine (SSZ) (1, 10, 100, 625 and 1000 mM). RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measured for (a) MMP-14, (b) MMP-15 and (c) MMP-16 by TaqMan low density array as previously described. Results shown are expressed as a fold induction of control (mean ± SD). Statistical significance was assessed where * $P<0.05$, ** $P<0.005$, *** $P<0.001$ using one-way ANOVA with bonferroni post-hoc test. (n=5)



6.4.6 The effect of sulfasalazine on the gene expression levels of the tissue inhibitors of metalloproteinases (TIMP) -1, -2, -3 and -4 in human articular chondrocytes.

In order to study the effect of sulfasalazine on gene expression levels of the tissue inhibitors of metalloproteinase, TIMP-1, -2, -3 and -4 in human articular chondrocytes, their expression was detected by the addition of cDNA to the TaqMan low density arrays as in section 6.3.2.

The measure of the level of gene expression in control sample was found to be 1.0 ± 0.01 for TIMP-1 (figure 6.6a), the addition IL-1α and OSM showed an increase in expression compared to control of 5.2 ± 0.8. Treatment of the cell with both IL-1α and OSM and sulfasalazine at a range of doses, showed that at the three highest doses of sulfasalazine (100, 625 and 1000 μM) the expression of TIMP-1 was significantly inhibited to 2.3 ± 0.6, 1.9 ± 0.3 and 1.7 ± 0.3 respectively. This suggests that due to the inhibition of MMPs such as MMP-1 by sulfasalazine, the cells are not required to express as much TIMP-1 in an attempt to block the effect of the MMPs.

The stimulation for human articular chondrocytes with IL-1α and OSM had no effect on the expression levels of TIMP-2, -3 or -4 and again the addition of sulfasalazine resulted in no significant reduction or induction of either TIMP-2, -3 or -4 expression.

6.4.7 The effect of sulfasalazine on the gene expression levels of aggrecanases ADAMTS -1, -4, -5 and -9 in human articular chondrocytes.

In order to quantify the effect of sulfasalazine on gene expression levels of the aggrecanases ADAMTS-1, -4, -5 and -9 during cartilage breakdown, their expression was detected by the addition of cDNA to the TaqMan low density arrays as in section 6.3.2.

In the experiment shown, the expression of ADAMTS-1 in control samples was shown to be 1.0 ± 0.3 (figure 6.7a). After stimulation with IL-1α and OSM a decrease was seen in the level of expression compared to control, which was found to be 0.9 ± 0.3, the addition of sulfasalazine to the stimulated cells showed a significant down regulation in the expression of ADAMTS-1, with the two highest concentration of sulfasalazine (625 and 1000μM) to 0.4 ± 0.1 and 0.3 ± 0.1 respectively.

The expression level of ADAMTS-4 in control human articular chondrocytes was measured and found to be 1.0 ± 0.5 (figure 6.7b). The stimulation of the cells with IL-1α and OSM, resulted in a significant increase in ADAMTS-4 gene expression compared to that of control, increasing the expression to 9.2 ± 2.7. The addition of Sulfasalazine to the

Figure 6.6 The effect of sulfasalazine on the gene expression levels of tissue inhibitors of metalloproteinases in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1/ OSM (0.02ng/ml)/(10ng/ml) for 24 hours in the presence of sulfasalazine (SSZ) (1, 10, 100, 625 and 1000 mM). RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measured for (a) TIMP-1, (b) TIMP-2, (c) TIMP-3 and (d) TIMP-4 by TaqMan low density array as previously described. Results shown are expressed as a fold induction of control (mean ± SD). Statistical significance was assessed where * $P<0.05$, ** $P<0.005$, *** $P<0.001$ using one-way ANOVA with bonferroni post-hoc test. (n=5)

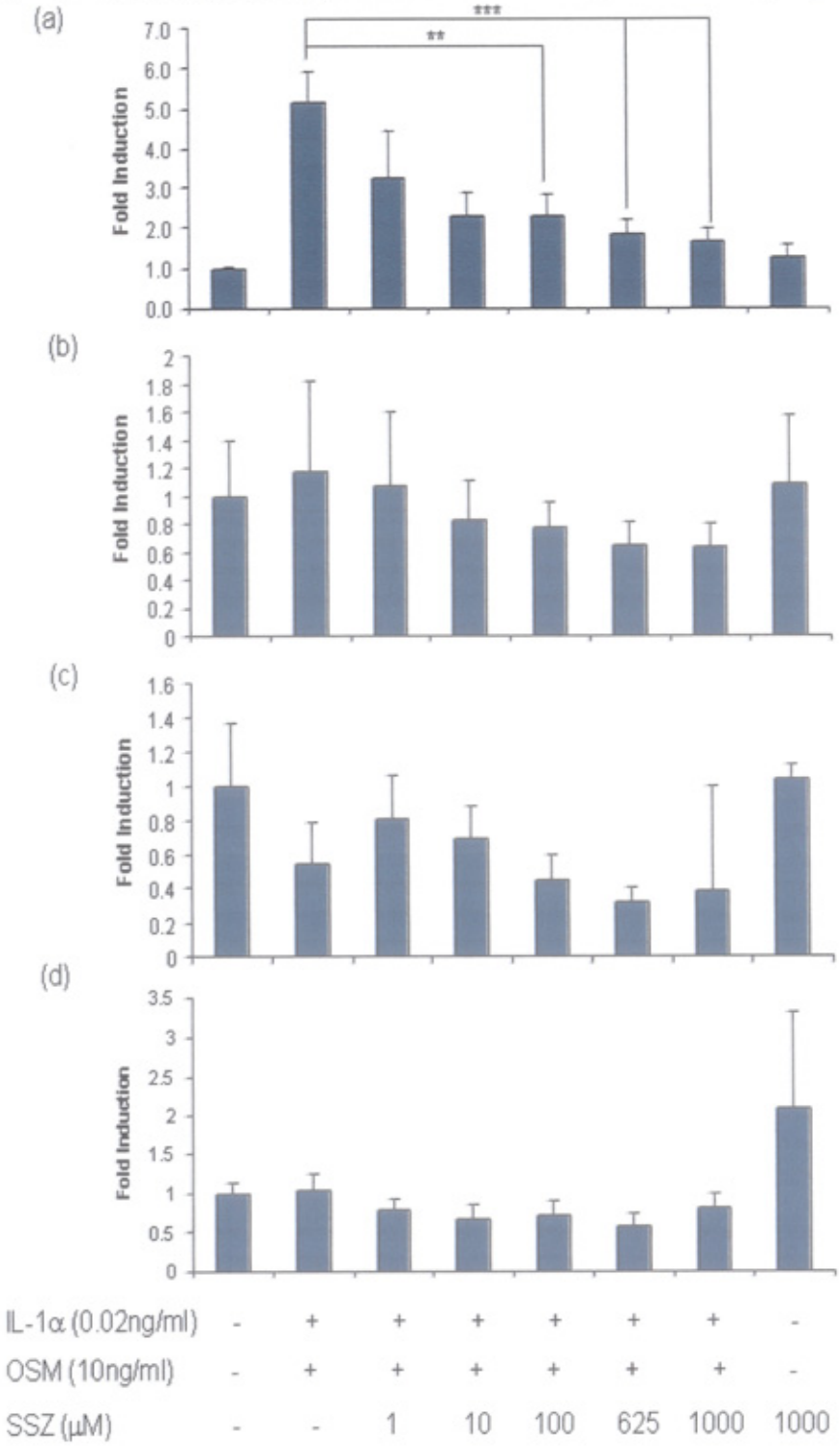
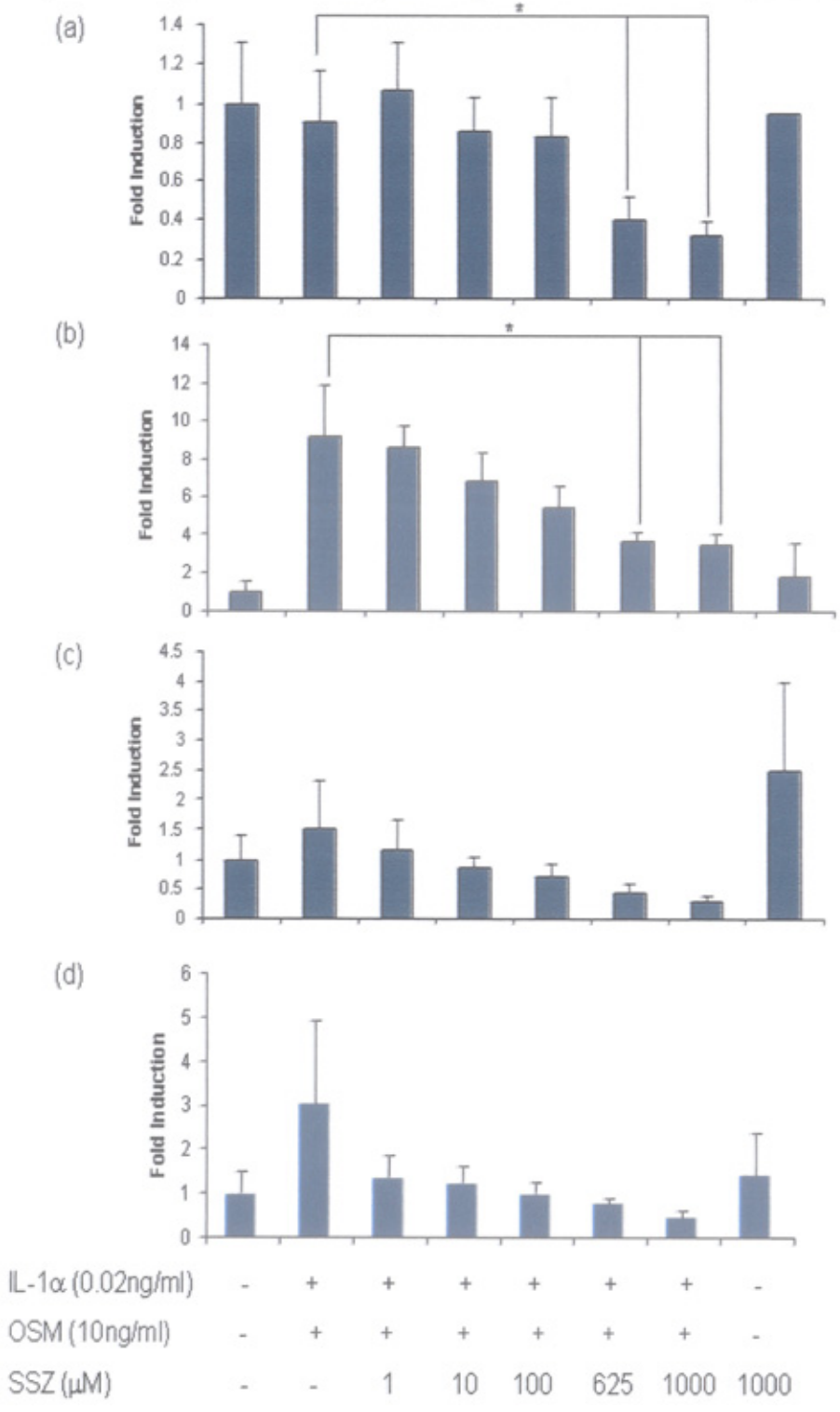


Figure 6.7 The effect of sulfasalazine on the gene expression levels of aggrecanases in human articular chondrocytes. Human articular chondrocytes were stimulated with IL-1 (0.02ng/ml) and OSM (10ng/ml) in combination for 24 hours in the presence of sulfasalazine (SSZ) (1, 10, 100, 625 and 1000 mM). RNA was extracted from the cells and reverse transcribed to cDNA. Relative gene expression was measured for (a) ADAMTS-1, (b) ADAMTS-4, (c) ADAMTS-5 and (d) ADAMTS-9 by TaqMan low density array as previously described. Results shown are expressed as a fold induction of control (mean ± SD). Statistical significance was assessed where * $P<0.05$, ** $P<0.005$, *** $P<0.001$ using one-way ANOVA with bonferroni post-hoc test. (n=5)



IL-1α and OSM stimulated human articular chondrocytes causes a significant down regulation in the expression level in a dose responsive manner with sulfasalazine at concentrations of 625 and 1000μM showing a significant decrease of 3.8 ± 0.4 and 3.6 ± 0.5 respectively.

In the experiment shown, stimulation of human articular chondrocytes with IL-1α and OSM showed an increase in the level of expression of ADAMTS-5 (figure 6.7c) and -9 (figure 6.7d). The treatment of the IL-1α and OSM stimulated cells with sulfasalazine caused no significant inhibition in the expression if ADAMTS-5 or -9.

6.5 DISCUSSION

In this chapter, the effect of adding sulfasalazine at a range of concentrations to IL-1 and OSM stimulated human articular chondrocytes on the gene expression of key enzymes implicated in the cartilage degradation that occurs in arthritis was investigated. This was assessed by using the TaqMan low density array technology for quantitative PCR. It has been well documented that the stimulation of human articular chondrocytes with IL-1 and OSM results in an increased expression at a gene level of key enzymes belonging to families such as matrix metalloproteinases (MMP), tissue inhibitors of metalloproteinases (TIMP) and a disintegrin and metalloproteinase domain with thrombospondin motifs (ADAMTS).

In this study, the stimulation of human articular chondrocytes with IL-1 and OSM was shown to cause an up regulation in the gene expression compared to basal level in MMP-1, -2, -3, -8, -9 and -13, TIMP-1, and ADAMTS-4, supporting previously published work.

In one publication it has been demonstrated that the stimulation of human articular chondrocytes by IL-1 and OSM results in a synergistic increase in the gene expression levels of MMP-1, -3, -8, -10, -12 and -13 (Barksby et al, 2006). Similarly in another study which investigated the gene expression of specific MMPs, TIMPs and ADAMTS in resorbing bovine cartilage IL-1 and OSM was shown to induce the expression of MMP-1, -2, -3, -9, -13 and -14, TIMP-1 and ADAMTS-4, -5 and -9 compared to basal levels, whilst MMP-16, TIMP-2 and -3 and ADAMTS-1 were found to be down regulated compared to basal levels (Milner et al, 2006).

As previously mentioned, very few studies have been published on the effect of sulfasalazine on gene expression in cells, with no study having been published on the affect of sulfasalazine on IL-1 and OSM stimulated chondrocytes or cartilage. Data from this chapter showing the affect of adding sulfasalazine to stimulated chondrocytes showed some interesting results. Firstly, the data clearly shows that sulfasalazine is able to block not only the up regulation of collagenases MMP-1 and -13, and gelatinase MMP-2 supporting data previously shown in chapter five, but it is also able to significantly inhibit the expression of collagenase MMP-8 and stromelysin MMP-3. Therefore it is possible, that it is this inhibitory effect of sulfasalazine on these cartilage degrading proteinases which ultimately leads to the apparent protective effect on cartilage shown for sulfasalazine.

The data in this chapter also clearly demonstrated that sulfasalazine is able to block aggrecan from the proteolytic attack as previously seen in chapter 5. While the inhibition of all the aggrecanases was observed, the most obvious effect came with ADAMTS-1 and -4, which were both significantly inhibited by sulfasalazine, suggesting that by inhibiting these key proteases, sulfasalazine is able to protect the interglobular domain of aggrecan.

The data in this chapter, shows that sulfasalazine is able to inhibit the effect TIMP regulation, with a clear and significant down regulation of TIMP-1 occurring when sulfasalazine is added to IL-1 and OSM stimulated cells compared to cells treated with IL-1 and OSM. This suggests that sulfasalazine is able to block the expression of TIMP-1 at a transcriptional level, where it is thought to be regulated and induced by growth factors and cytokines. With this in mind sulfasalazine may inhibit the up regulation of TIMP-1 expression by interfering with the modulation of the cytokine pathways involved in the MMP/ TIMP balance.

It is clear from the results shown in this chapter, that sulfasalazine is able to block the effect of cartilage degradation by targeting and ultimately blocking the effect of key proteases involved in the breakdown of cartilage.

Summary

Results in this chapter have shown that:

- Sulfasalazine is able to inhibit the gene expression of key MMPs in the collagenase, gelatinase and stromelysin families.
- Sulfasalazine is able to suppress the up regulation of aggrecanases at the gene level.

- Sulfasalazine is also able to block the expression of TIMP-1.

CHAPTER 7: The effect of sulfasalazine on signalling mechanisms

7.1 INTRODUCTION

Data in previous chapters have shown the potential of sulfasalazine as a chondroprotective drug, due to its ability to block cartilage destruction, preventing the release of proteoglycan and collagen from explant cartilage and by its down regulation of key inflammatory enzymes in stimulated human articular chondrocytes, showing many novel findings. On this basis it was decided that further investigation of its specific mechanism was required. The study of arthritis in animals has strongly highlighted a role for interleukin-1 in joint damage, with the injection of interleukin-1 into the joints of rabbits resulting in the loss of cartilage matrix (Pettipher et al, 1986). Interleukin-1 signalling is known to be highly complex, with interleukin-1 binding to two types of cell surface receptors (Type I and Type II). However, only type I is capable of leading to intracellular signalling. Interleukin-1 is known to cause the activation of a number of signal transduction pathways, which engage in a high level of cross-talk. Signal transduction pathways closely involved in inflammation include the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor kappa B (NF-κB) pathway.

As previously mentioned in section 1.4.1, NF-κB has been shown to be crucial in the induction of inflammatory responses, with the expression of NF-κB being increased at sites of inflammation such as in rheumatoid synovium. Studies of rheumatoid synovium have shown NF-κB expression within the nuclei of synovial cells (Tak and Firestein, 2001), whilst another study examining synovial tissue from patients with rheumatoid arthritis and osteoarthritis showed greater levels of NF-κB binding to DNA in rheumatoid synovium to that of synovium for osteoarthritis patients (May and Ghosh, 1998). This suggests that there is a much higher level of cytokine release occurring in rheumatoid arthritis than in osteoarthritis.

The MAPK pathways as previously mentioned in section 1.5.2 are activated in response to inflammatory cytokines and are thought to be involved in both osteo- and rheumatoid arthritis. The three MAPK families which include ERK, JNK and P38, control the activation of transcription factors such as activator protein-1 (AP-1). They are expressed in rheumatoid synovium and are thought to control the synthesis of inflammation mediators such as the metalloproteinase responsible for cartilage degradation (Herlaar and

Brown, 1999; Berenbaum et al, 2003). Transcription factor, AP-1 has an essential role in cartilage and bone development. A number of studies suggest that AP-1 regulates cartilage and bone development, for example, AP-1 activation mediates the Wnt regulation of chondrogenesis (Tufan et al, 2002) and regulates hypertrophic maturation of chondrocytes (Moritani et al, 2003). AP-1 also plays an essential role in chondrocyte differentiation through the induction of c-fos protein expression (Ionescu et al, 2001). As well as the regulation of chondrocyte differentiation, it is also thought that AP-1 may regulate the destruction of articular cartilage, and the inhibition of IL-1β stimulated AP-1 down regulates matrix metalloproteinase expression in articular chondrocytes (Liacini et al, 2002; Hwang et al 2005).

Sulfasalazine is a drug which has been used for many years in the treatment of diseases such as rheumatoid arthritis and inflammatory bowel disease. It is well known that sulfasalazine is a selective inhibitor of NF-κB activation, by inhibiting the translocation of NF-κB to the nucleus, which is paralleled by preventing the translocation of IκB to the proteosome (Wahl et al, 1998; Oakley et al, 2005). Whilst sulfasalazine's effect on the NF-κB pathway has been well documented, its affect on other pathways has been largely ignored. However, one study by Elsharkawy et al, looking at the effects of sulfasalazine on hepatic stellate cells looked into reports which suggested that the induction of growth arrest and DNA damage-inducible beta (Gadd45β) (a member of a family of genes which are involved in the regulation of cell cycle progression and apoptosis induction) by NF-kB is able to block JNK induced apoptosis by inhibiting MKK7 activation of JNK (Papa et al, 2004). Thus suggesting that NF-κB protects the hepatic stellate cells from apoptosis and accelerates recovery from liver fibrosis; it was also suggested that this effect may be due to sulfasalazine inhibiting the activation of AP-1 (Elsharkawy et al, 2005).

7.2 AIMS

The aims of this chapter are to investigate the effect of sulfasalazine on key signalling pathways including the NF-kB and mitogen-activated protein kinase pathway.

7.3 METHODS

7.3.1 Cell culture

Human articular chondrocytes were derived from articular cartilage obtained from consented patients following surgery. Enzymatic digestion of tissue and maintenance and culture of cells were as previously described in section 2.5. After the addition of test reagents cells were incubated for 24 hours and the medium harvested for assay. All conditions were assayed in triplicate and primary chondrocytes were used at low passage (passage number 1).

7.3.2 Real Time PCR

Extracted primary human articular chondrocytes were incubated with and without the combination of IL-1α [1 ng/ml] + OSM [10 ng/ml). RNA was extracted from the cells, reverse transcribed and measured for gene expression as described in section 2.6.

7.3.3 Cytoplasmic and Nuclear Fraction Extraction

Extracted primary human articular chondrocytes were incubated with and without the combination of IL-1α [1 ng/ml] + OSM [10 ng/ml) either alone, or in the presence of sulfasalazine [1 – 1000 μM]. Cytoplasmic and nuclear fractions were extracted from the cells as described in section 2.10.

7.3.4 Bradford Assay

Cytoplasmic and nuclear fractions previously extracted as described in section 7.3.3, were analyzed for protein concentration by Bradford assay as described in section 2.11.

7.3.5 PANARAMA Antibody Microarray

Extracted primary human articular chondrocytes were incubated with and without the combination of IL-1α [1 ng/ml] + OSM [10 ng/ml) either alone, or in the presence of sulfasalazine at 1 μM and 1000 μM. Total cell extracts were extracted from the cells, labelled with Cy3 and Cy5 fluorescent markers and assayed as described in section 2.9.

7.3.6 Western Blotting

Cytoplasmic and nuclear fractions previously extracted as described in section 7.3.3, were analyzed by western blot as describe in section 2.8.

7.4 RESULTS

7.4.1 Experiment to investigate the expression of chondrocyte specific markers when passaging human articular chondrocytes.

In order to determine if chondrocyte specific markers were still expressed following the passaging of human articular chondrocytes and if sufficient protein was obtained for western blot analysis, primary human articular chondrocytes were passaged from passage 0 to passage 3 and grown to ~80% confluence.

7.4.1.1 The effect of sub-culturing primary human articular on changes in cell shape.

Human articular chondrocytes were digested from human articular cartilage and plated into a 6-well tissue culture plastic plate and T75 flasks, and the cells were grown to ~80% confluence. Once sufficient confluence was achieved the 6-well plate was serum starved for 24 hours, before stimulation with and without IL-1α + OSM (1/10 ng/ml). Phase contrast images were then taken of the cells in order to look at changes in cell shape (figure 7.1). The T75 flasks were then taken and sub-cultured into a 6-well plate and another T75 flask, this process was continued until passage 3 was reached. The cells at passage 0 were seeded at a high confluence of 250,000 cells per well, the cells remain round after adherence and started to spread out as they reach ~80% confluence, however maintaining their round shape. The cells at passage 1 and 2 after adherence remained round for approximately 24 hours after plating, before beginning to spread out on the plastic although still maintaining the same shape seen at passage 0. At passage 3, the cells appeared to take longer to adhere to the culture plastic and once doing so they began to spread out. The cells were not as rounded as seen in the earlier passages with an increasing tendency to adopt a flattened spindle like shape.

7.4.1.2 The effect on changes in the expression of genes known to be key in the phenotyping of chondrocytes: collagen type I, collagen type II and SOX-9.

Passaged cells used in section 7.4.1.1, were lysed and the RNA extracted using Qiagen RNeasy columns. The RNA was reverse transcribed and analyzed by real-time PCR for collagen type I, collagen type II and SOX-9 (figure 7.2) and expressed as relative quantification ($x10^6$) (RQ). The expression of collagen type I (Coll1A) was seen to be expressed relatively high in passage 0 and 1 chondrocytes 0.28 ± 0.01 RQ and 0.22 ± 0.04 RQ respectfully; however the expression of Coll1A was significantly reduced at passages 2

Figure 7.1 Phase contrast images showing changes in cell shape of human articular chondrocytes through an number of passages.

Human articular chondrocytes where grown to confluence before being passaged. At each passage step (P0, P1, P2 and P3) cells were stimulated with or without IL-1α + OSM (1 + 10 ng/ml). Images shown at a scale of 100 micron.

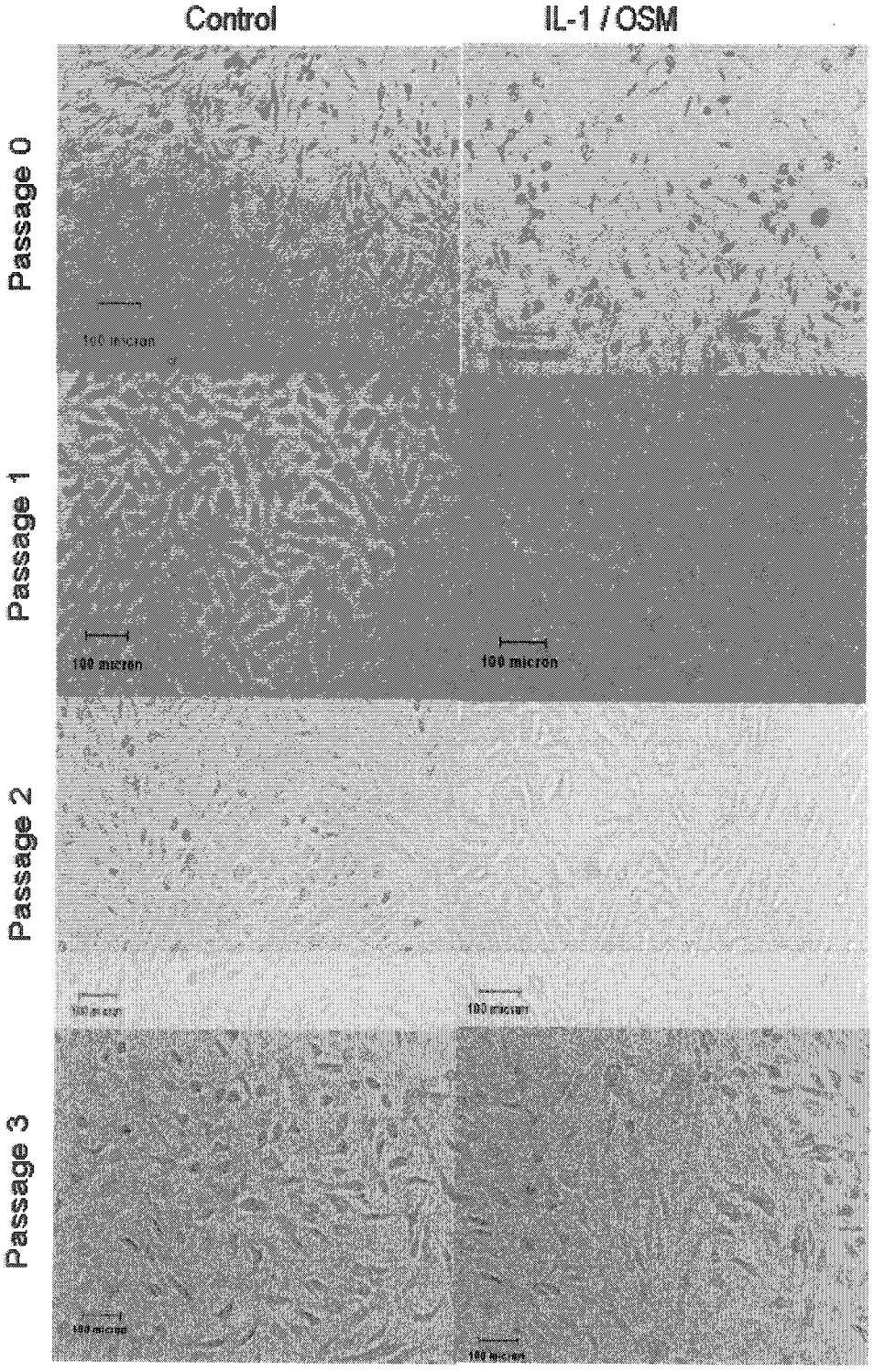
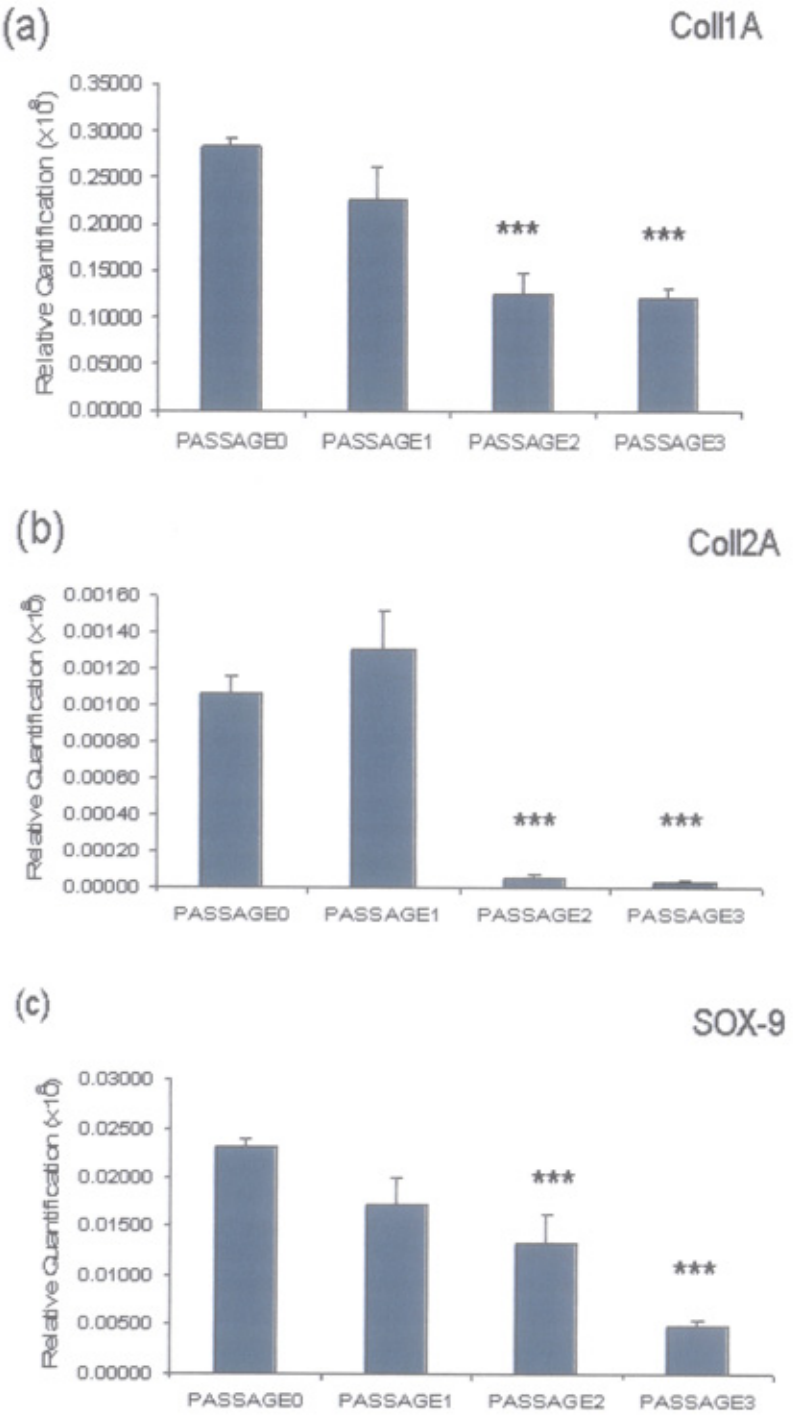


Figure 7.2 Expression of Collagen Type 1, Collagen Type 2 and SOX-9 in passaged human articular chondrocytes.

Human articular chondrocytes where grown to confluence before being passaged. At each passage step (P0, P1, P2 and P3) cells. RNA was extracted, reverse transcribes and cDNA expression of (a) collagen 1, (b) collagen 2 and (c) Sox-9 was measured by real-time PCR. *** $P<0.001$



(0.13 ± 0.02 RQ) and 3 (0.12 ± 0.01 RQ). No significant change was seen in cells stimulated with IL-1 + OSM. The expression of collagen type II (Coll2A) was also highly expressed at passage 0 and 1 in control and IL-1 + OSM stimulated cells. At passage 2 and 3, the expression of Coll2A was significantly down regulated in control and IL-1 + OSM samples. As with collagen type I and collagen type II, SOX-9 is also highly expressed both in control and IL-1 + OSM stimulated samples at passage 0. However a gradual downward trend is seen through passage 1 to 3 in control and IL-1 + OSM stimulated cells, with expression levels for both treatments being significantly reduced at passage 2 and 3 (data not shown). With the ratio of collagen type II to collagen type I being 0.0038 at passage 0 and 0.0058 at passage 1, this data suggests that the expression of genes which are key in the phenotype of chondrocytes begin to reduce in their levels of expression after passage 1, therefore it would be advisable that cells should only be passaged once if sub-culturing is necessary.

7.4.2 The effect of sulfasalazine on the degradation of IκB.

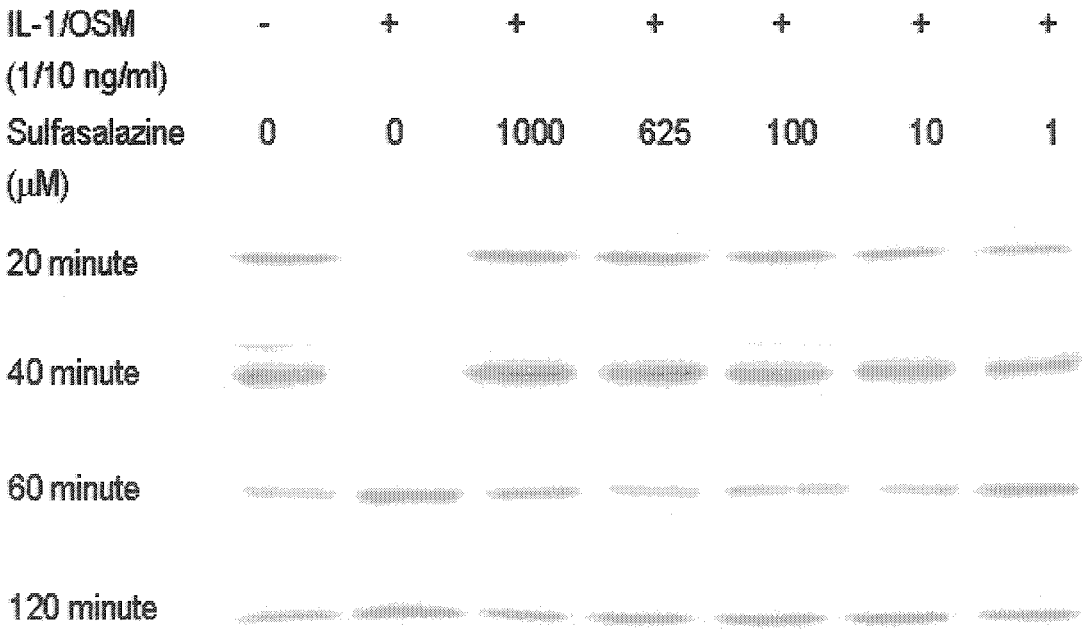
Human articular chondrocytes were grown to confluence before being sub-cultured. The cells were then allowed to grow to ~80% confluence, before being serum starved for 24 hours. The cells were then treated with and without IL-1 + OSM (1/10 ng/ml) in the presence of sulfasalazine at 1 – 1000 μM for the following time points 20, 40, 60 and 120 minutes. Cytoplasmic fractions were then extracted for each sample and protein concentration measured. The samples were all equalised to ensure equal protein loading. The samples were then run via western blot and probed to investigate IκB activity. IκB was found to be present in control samples throughout all the time points. After stimulation with the IL-1 + OSM for 20 and 40 minutes, IκB appeared to have degraded with no bands being present, however by 60 minutes of stimulation, IκB reappeared and still remained after 120 minutes of stimulation. After the IL-1 + OSM stimulated HACs were treated with sulfasalazine, IκB was seen to be highly expressed at all time points.

7.4.3 Experiment to screen the mitogen-activating protein kinase pathways (MAPK) by PANARAMA signalling microarray for possible effects by sulfasalazine.

Human articular chondrocytes were grown to confluence before being sub-cultured. The cells were then allowed to grow to ~80% confluence, before being serum starved for 24 hours. The cells were then treated with and without IL-1 + OSM (1/10 ng/ml) in the

Figure 7.3 The effect of sulfasalazine on the degradation of IκB to the proteosome

Human articular chondrocytes were stimulated with IL-1(1 ng/ml) + OSM (10 ng/ml) in the presence of sulfasalazine (1 – 1000 mM) for 20, 40, 60 and 120 minutes. cytoplasmic fractions were collected analyzed for IκB-α by western blot



presence of sulfasalazine at 1 μM and 1000 μM for 20 minutes. Total cell extracts were then harvested for each sample and processed as described in section 2.9.

Table 7.1 Table of Panorama signalling microarray antibodies investigated.

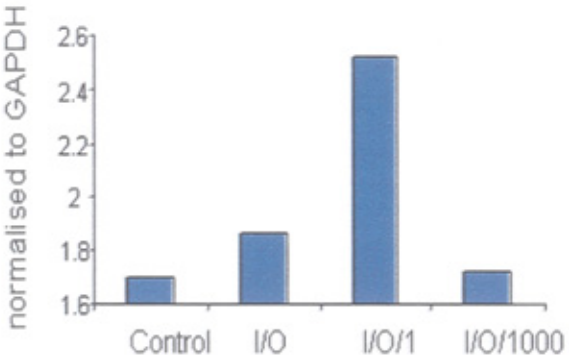
Antibody	SIGMA No.	Poly/ Monoclonal	Human reactivity
ERK-1	M7927	Polyclonal	Y
ERK-, ERK-2	M5670	Polyclonal	Y
Non-Phosphorylated ERK	M3807	Monoclonal	Y
MAPKAPK-2	M3550	Polyclonal	Y
Monophosphorylated Threonine	M3557	Monoclonal	Y
Phosphorylated ERK-1 & -2	M7802	Monoclonal	Y
MKK4	M0422	Polyclonal	Y
Monophosphorylated Tyrosine	M3682	Monoclonal	Y
MKP-1	M3787	Polyclonal	Y
Diphosphorylated ERK-1 & -2	M9692	Monoclonal	Y
ERK-2	M7431	Monoclonal	Y
MEK	M5795	Polyclonal	Y
Non-activated p38	M8432	Monoclonal	Y
P38	M0800	Polyclonal	n/d
Diphosphorylated p38	M8177	Monoclonal	Y
JNK1/2	J4500	Polyclonal	Y
Diphosphorylated JNK	J4750	Monoclonal	Y
Phosphor-c-Jun ($pSer^{63}$)	J2128	Polyclonal	Y
Phosphor-c-Jun ($pSer^{73}$)	J2253	Polyclonal	Y
AP-1	A5968	Polyclonal	Y
ERK-5	E1523	Polyclonal	Y
RAF-1	R5773	Polyclonal	Y
RAF-1	R2404	Monoclonal	Y
VEGFR-1	V4762	Monoclonal	Y
ATF-1	A7833	Polyclonal	Y
ATF-2	A4086	Polyclonal	Y
Phosphor-ATF-2	A4095	Monoclonal	Y
RSK-1	R5145	Polyclonal	Y
GAPDH	G8795	Monoclonal	Y
SIRPa1	S1311	Polyclonal	n/d
Sos1	S2937	Polyclonal	Y
SAPK3	S0315	Polyclonal	Y
SKK2	S5308	Polyclonal	Y
MEKK4	M7194	Monoclonal	Y

In this experiment, the microarrays which are designed to screen the MAPK pathways ERK, JNK and P38 were used to investigate the effects of sulfasalazine on these pathways (Table 7.1). Four different conditions were selected: control, IL-1 + OSM (1/10 ng/ml),

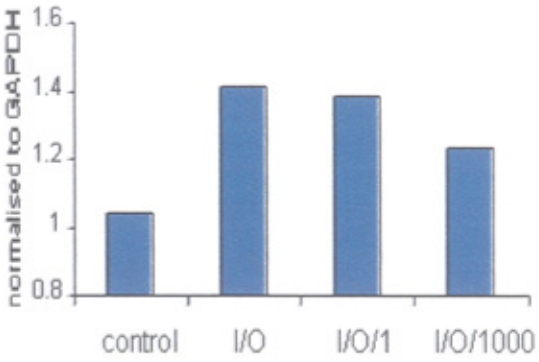
Figure 7.4 The effect of sulfasalazine on the mitogen-activated protein kinases pathway using the PANARAMA microarray

Human articular chondrocytes were stimulated with and without IL-1(1 ng/ml) + OSM (10 ng/ml) in the presence of sulfasalazine at 1 mM and 1000 mM for 20 minutes. Total cell extracts were collected and analyzed via the PANARAMA signalling micro array. Results were normalised to GAPDH.

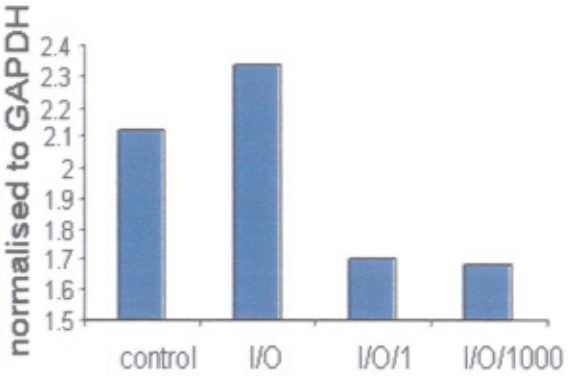
(a) MKK4



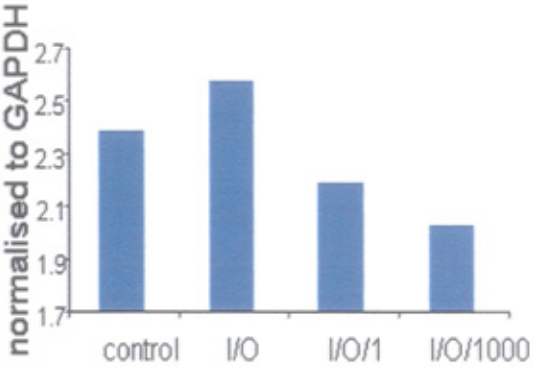
(b) JNK



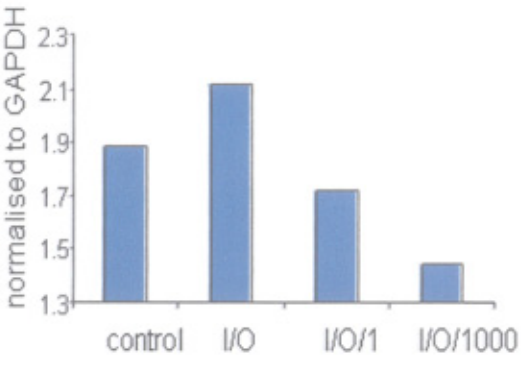
(c) ATF-2



(d) phosph-ATF-2



(e) AP-1



IL-1 + OSM with sulfasalazine at 1 μM and IL-1 + OSM with sulfasalazine at 1000 μM. The microarrays were scanned by use of a microarray scanner and the results calculated as a ratio between the Cy3 / Cy5 values for each antibody, these results were then normalised to GAPDH. A number of key observations were made (figure 7.4). The phosphorylation of MAPK kinase kinase 4 (MKK4) (figure 7.4a) was increased after stimulation with IL-1 + OSM compared to control; the addition of sulfasalazine at 1000 μM appeared to cause this effect to be down regulated, whilst sulfasalazine at 1 μM caused the phosphorylation of MKK4 to be accentuated. Jun-amino-terminal kinase (JNK) (figure 7.4b) was down regulated by the addition of sulfasalazine at 1000 μM to IL-1 + OSM stimulated cells compared to IL-1 + OSM alone; sulfasalazine at 1 μM had no effect. Sulfasalazine also showed an effect with pan-ATF2 (figure 7.4c) and phosphor-ATF-2 (figure 7.4d), where both concentrations appeared to suppress the up regulation of both pan and phospho-AFT-2 seen after stimulation with IL-1 + OSM. An effect was also seen with AP-1, where the stimulation of the chondrocytes with IL-1 + OSM caused the activation of AP-1, however the addition of sulfasalazine at both concentrations to IL-1 + OSM appeared to have an inhibitory effect on AP-1 activation. The effects described above suggest that sulfasalazine may in fact have an effect on a specific area of the MAPK pathway by ultimately blocking the activation of AP-1. No other significant results were seen with the signalling microarray.

7.4.4 An experiment to investigate effects by sulfasalazine on the JNK signalling in the MAPK pathway.

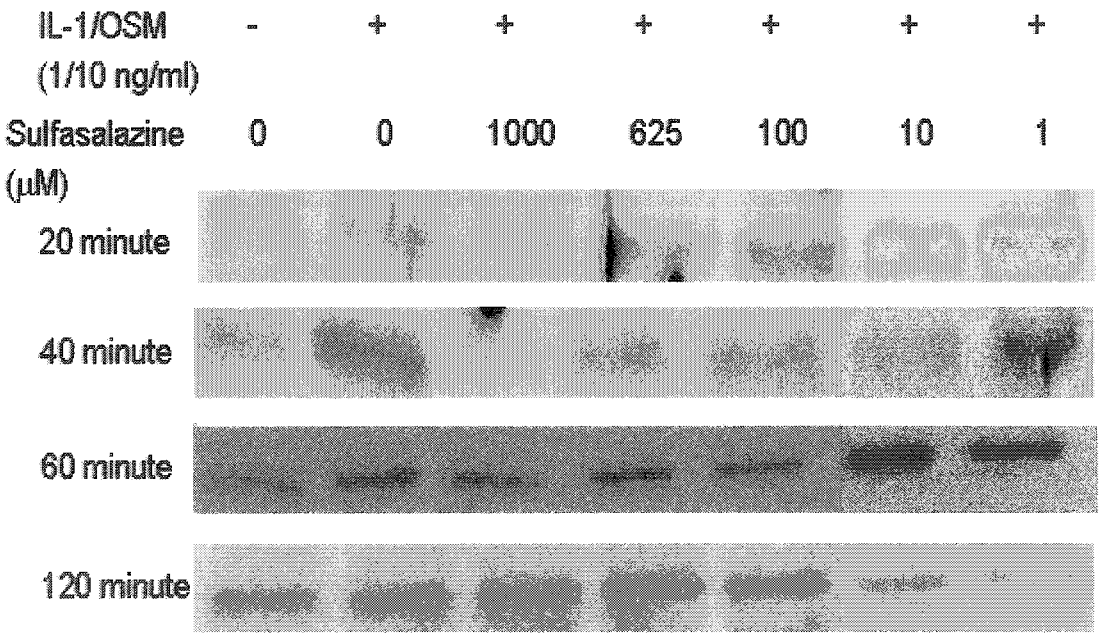
Human articular chondrocytes were grown to confluence before being sub-cultured once. The cells were then allowed to grow to ~80% confluence, before being serum starved for 24 hours. The cells were then treated with and without IL-1 + OSM (1/10 ng/ml) in the presence of sulfasalazine at 1 – 1000 μM for the following time points 20, 40, 60 and 120 minutes and cytoplasmic and nuclear fractions were collected in order to confirm results seen in section 7.4.3 with the protein signalling microarray.

7.4.4.1 The effect of sulfasalazine on phosphorylated MAPK kinase kinase 4 (MKK4)

Cytoplasmic fractions were prepared and a western blot was carried out to detect phospho-MKK4 (figure 7.5). After 20 minutes of stimulation with IL-1 + OSM, the phosphorylation of MKK4 could clearly been seen, whilst the addition sulfasalazine at

Figure 7.5 The effect of sulfasalazine on MAPK kinase kinase 4 phosphorylation

Human articular chondrocytes were stimulated with IL-1(1 ng/ml) + OSM (10 ng/ml) in the presence of sulfasalazine (1 – 1000 mM) for 20, 40, 60 and 120 minutes. Cytoplasmic fractions were collected analyzed for MKK4 by western blot. The experiment was repeated twice and the best representative blot used below.



1000 μM inhibited this effect. However lower doses of sulfasalazine had no effect on inhibiting the phosphorylation of MKK4. After 40 minute stimulation with IL-1 + OSM, the phosphorylation of MKK4 could clearly been seen, whilst the addition of sulfasalazine at 100 – 1000 μM inhibited this effect. At both the 60 and 120 minute stimulation sulfasalazine did not block the phosphorylation of MKK4. This suggests that sulfasalazine at 1000 μM may be able to block MKK4 phosphorylation supporting the microarray data shown above.

7.4.4.2 The effect of sulfasalazine on the activation and phosphorylation of Jun-amino-terminal kinase (JNK1/2/3)

Cytoplasmic fractions were prepared and a western blot carried out to investigate the activation and phosphorylation of JNK1/2/3 (figure 7.6). In control samples the presence of JNK1 could be clearly seen, whilst JNK2/3 could only be seen in control samples at 40, 60 and 120 minute time points. After 20 minutes of stimulation with IL-1 + OSM, the activation and phosphorylation of JNK 2/3 had occurred, whilst no effect was seen with JNK1 activation. However after 40 minutes of stimulation activation of JNK1/2/3 had occurred and this was again seen after 60 and 120 minutes of stimulation. The addition of sulfasalazine to the stimulated cells showed no effect on the activation of JNK1 after 20 minutes but JNK2/3 was clearly seen to be inhibited at concentrations of 100, 625 and 1000 μM. However after 40 minutes inhibition of JNK1/2/3 was seen. After the 60 and 120 minutes stimulation sulfasalazine failed to block the activation and phosphorylation of JNK1/2/3. These results suggest that the inhibition of JNK1/2/3 activation and therefore its further phosphorylation may be blocked by sulfasalazine and is in agreement with the data seen with the signalling microarray (section 7.4.3).

7.4.4.3 The effect of sulfasalazine on the phosphorylation and activation of transcription factor AFT-2

Nuclear fractions were prepared from the experiment as described in section 7.4.4. A western blot carried out to investigate the phosphorylation and activation of ATF-2 using both pan AFT-2 and phospho-ATF-2 antibodies (figure 7.7). After 20 minutes of stimulation with IL-1 + OSM, the western blot showed that phosphorylation of ATF-2 was beginning to take place with a band being visible with both the pan and phospho antibodies. The presence of sulfasalazine with IL-1 + OSM appeared to be an inhibiting

Figure 7.6 The effect of sulfasalazine on the phosphorylation of JNK1/2/3.
Human articular chondrocytes were stimulated with IL-1(1 ng/ml) + OSM (10 ng/ml) in the presence of sulfasalazine (1 – 1000 mM) for 20, 40, 60 and 120 minutes. Cytoplasmic fractions were collected analyzed for phospho-JNK by western blot.

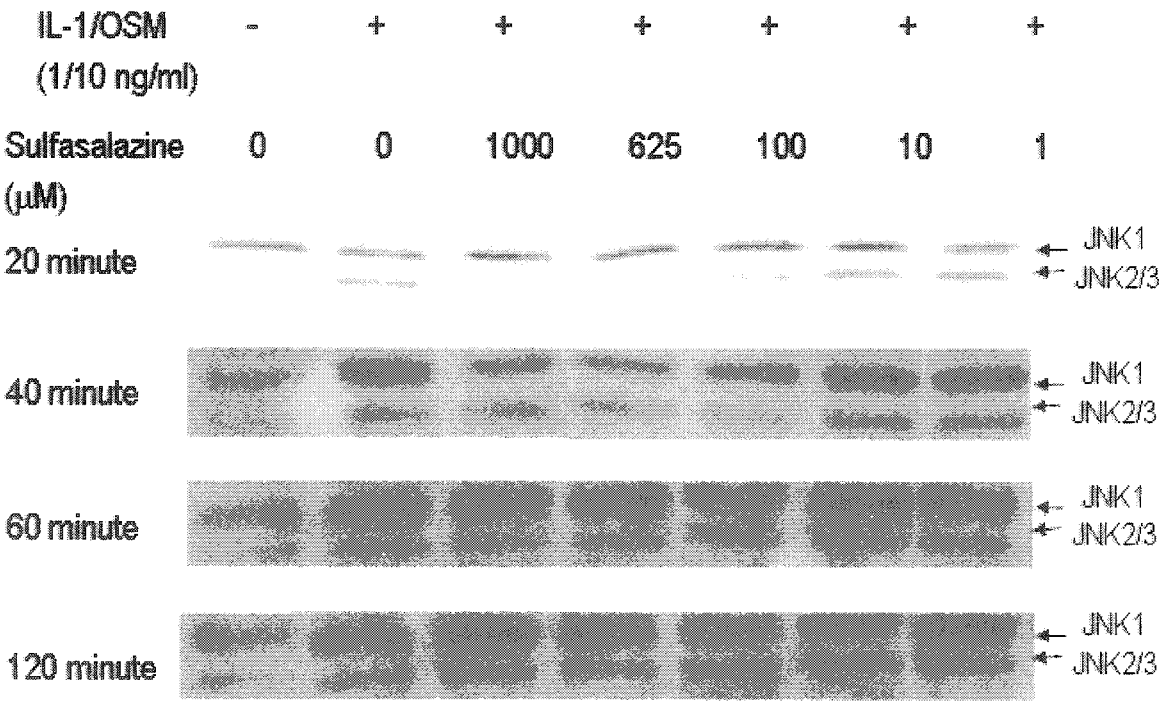
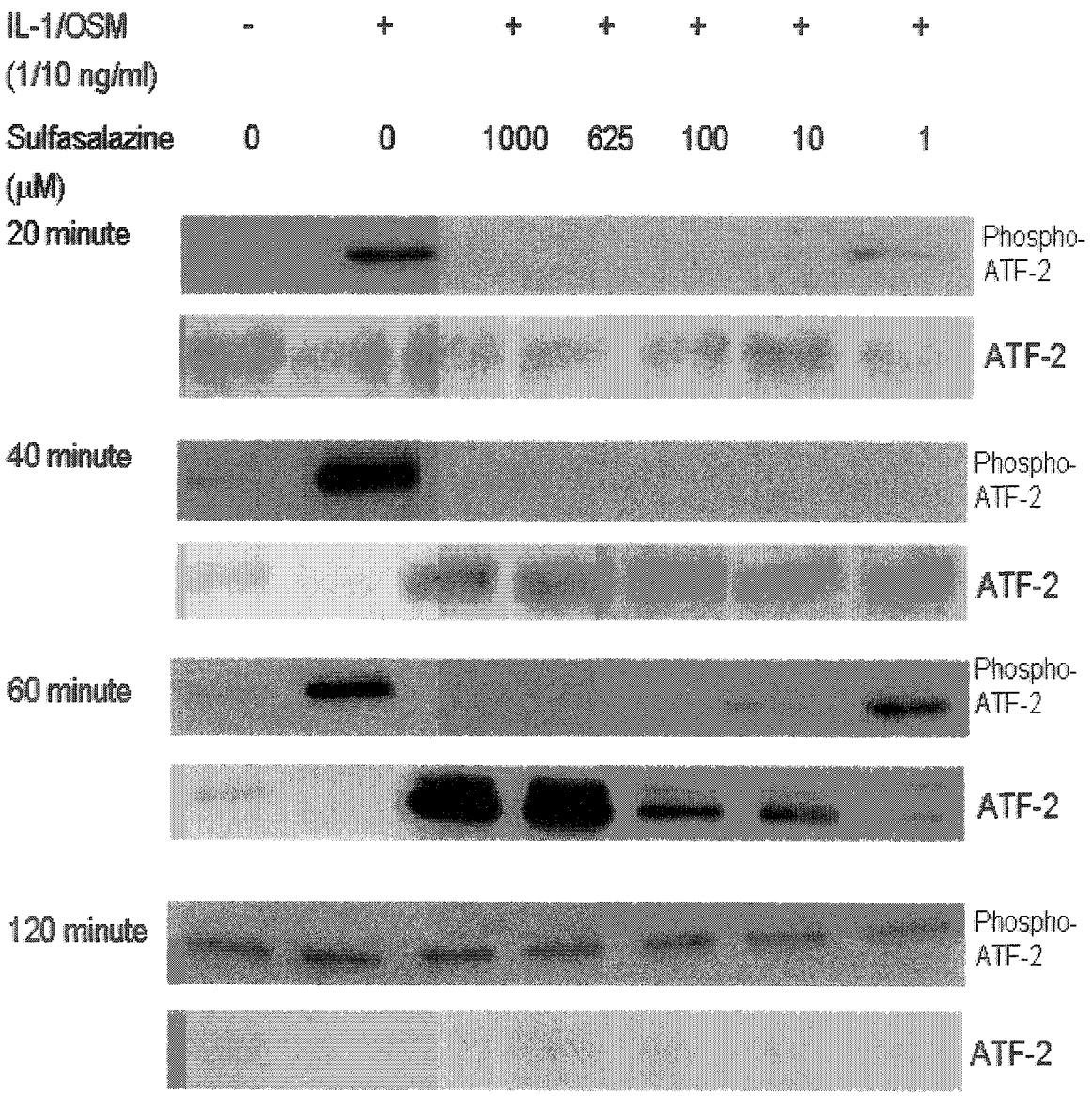


Figure 7.7 The effect of sulfasalazine on the phosphylation and activation of ATF-2.

Human articular chondrocytes were stimulated with IL-1(1 ng/ml) + OSM (10 ng/ml) in the presence of sulfasalazine (1 – 1000 mM) for 20, 40, 60 and 120 minutes. Nuclear fractions were collected analyzed for pan-ATF-2 an phospho-ATF-2 by western blot



event. After 40 minutes of stimulation no band was present with the pan antibody whilst with the phospho-ATF-2 antibody IL-1 + OSM stimulation had caused phosphorylation of ATF-2 to take place. No bands were visible with the phosphor-ATF-2 antibody after the treatment of IL-1 + OSM with sulfasalazine, whilst bands clearly remain after probing with the pan-ATF-2 antibody. This effect was again seen after the 60 minute time point, although phospho-ATF-2 bands were beginning to come visible with sulfasalazine at 1 and 10 μM. After the two hour time point, sulfasalazine was unable to block the phosphorylation and activation of ATF-2, with bands being present after probing with the phospho antibody and only faint bands present after probing with the pan antibody. This data again supports results seen with the microarray.

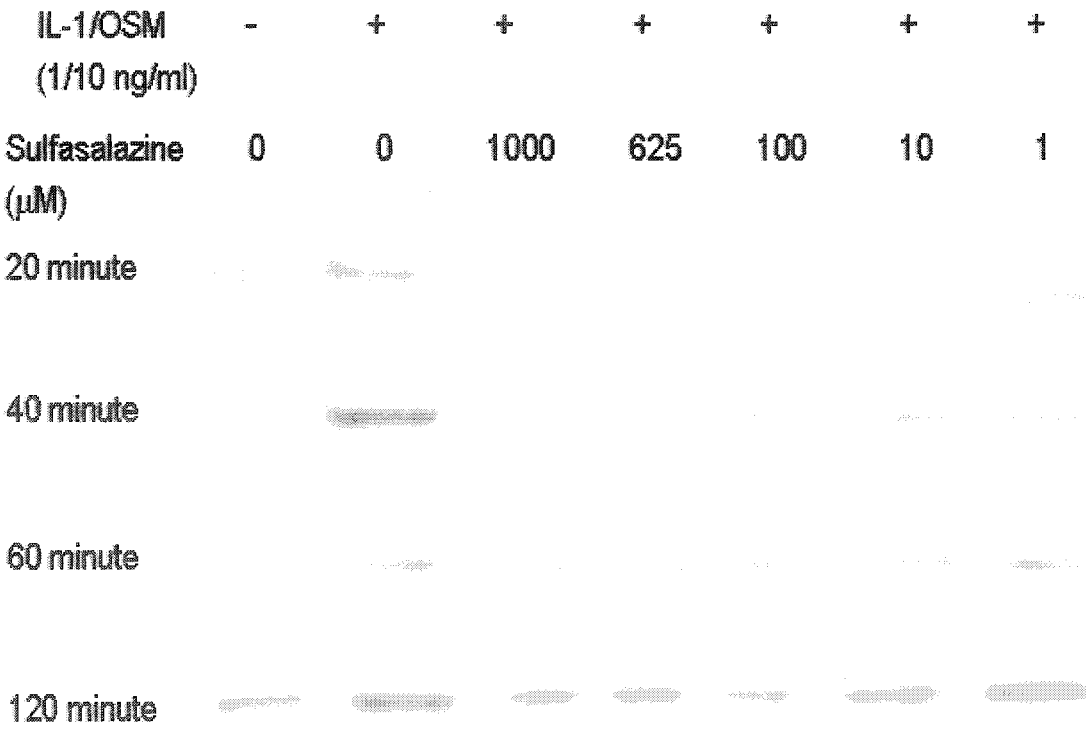
7.4.4.4 The effect of sulfasalazine on the activation of AP-1

Nuclear fractions were taken from the experiment as described in section 7.4.4. A western blot carried out to investigate the activation of AP-1 (figure 7.8). After 20 minutes of stimulation with IL-1 + OSM, the western blot showed that the activation of AP-1 was beginning to take place with faint bands being visible. The addition of sulfasalazine with IL-1 + OSM shown no bands and appeared to be blocking activation occurring. After 40 minutes of stimulation with IL-1 + OSM AP-1 activation had taken place with a band being clearly visible, whilst there only faint bands after treatment with sulfasalazine. After 60 minutes of stimulation activation of AP-1 was still taking place with IL-1 + OSM alone, whilst bands were also visible with sulfasalazine at concentrations of 1, 10 and 100 μM. After 120 minutes of stimulation with IL-1 + OSM, sulfasalazine was unable to block the activation of AP-1. This data suggests that sulfasalazine is able to block the activation of AP-1, supporting data seen in section 7.4.3.

7.5 DISCUSSION

In this chapter, the effect of sulfasalazine on the cellular mechanisms leading to arthritis was investigated using human articular chondrocytes. In order to ensure sufficient protein was obtained in the cytoplasmic and nuclear fractions for detection by western blot, it was necessary to passage the chondrocytes. The phenotype of chondrocytes is characterised by the expression of specific genes such as collagen type II and transcription factor SOX-9 (Zhao et al, 1997). Collagen type II is known to be abundant in articular cartilage

Figure 7.8 The effect of sulfasalazine on the activation of AP-1
Human articular chondrocytes were stimulated with IL-1(1 ng/ml) + OSM (10 ng/ml) in the presence of sulfasalazine (1 – 1000 mM) for 20, 40, 60 and 120 minutes. Nuclear fractions were collected analyzed for AP-1 by western blot



(Buckwalter et al, 2004), whilst SOX-9 is expressed in cartilage and thought to control a number of genes in articular cartilage. The expression of SOX-9 is known to rapidly decline in chondrocytes in monolayer cultures which is coupled by a reduction in collagen type II (Stokes et al, 2001; Hardingham t al, 2002; Tew et al, 2007). As cells are passaged their expression continues to drop. Tests were carried out to ensure the cells were still phenotypically chondrocytic after passaging by measuring the expression of SOX-9, collagen type II and collagen type I, throughout a number of passages. The results of these tests suggest that whilst no significant change was seen in the expression of collagen type II and SOX-9 at passage 1, the rapid decrease seen in the expression of these genes by passage 2 and 3 highlights possible changes in the chondrocytic phenotype. Interestingly, the expression of collagen type I was found to be unexpectedly high at both passage 0 and passage 1 and again a reduction was seen in expression levels at passage 2 and passage 3. From the results obtained it was therefore decided that cells would be used at passage 1 for the mechanistic experiments.

A number of signalling pathways are known to be involved in the disease process of arthritis. Both AP-1 and NF-κB have been linked with rheumatoid arthritis with both being markedly increased (Han et al, 1998). NF-κB had been shown to be present in the synovium of patients with rheumatoid arthritis and osteoarthritis (Morel and Berenbaum, 2004), whilst AP-1 has been shown in rheumatoid synovium (Asahara et al, 1997) and it is thought to be involved in articular cartilage destruction within the joint (Shiozawa et al, 1997).

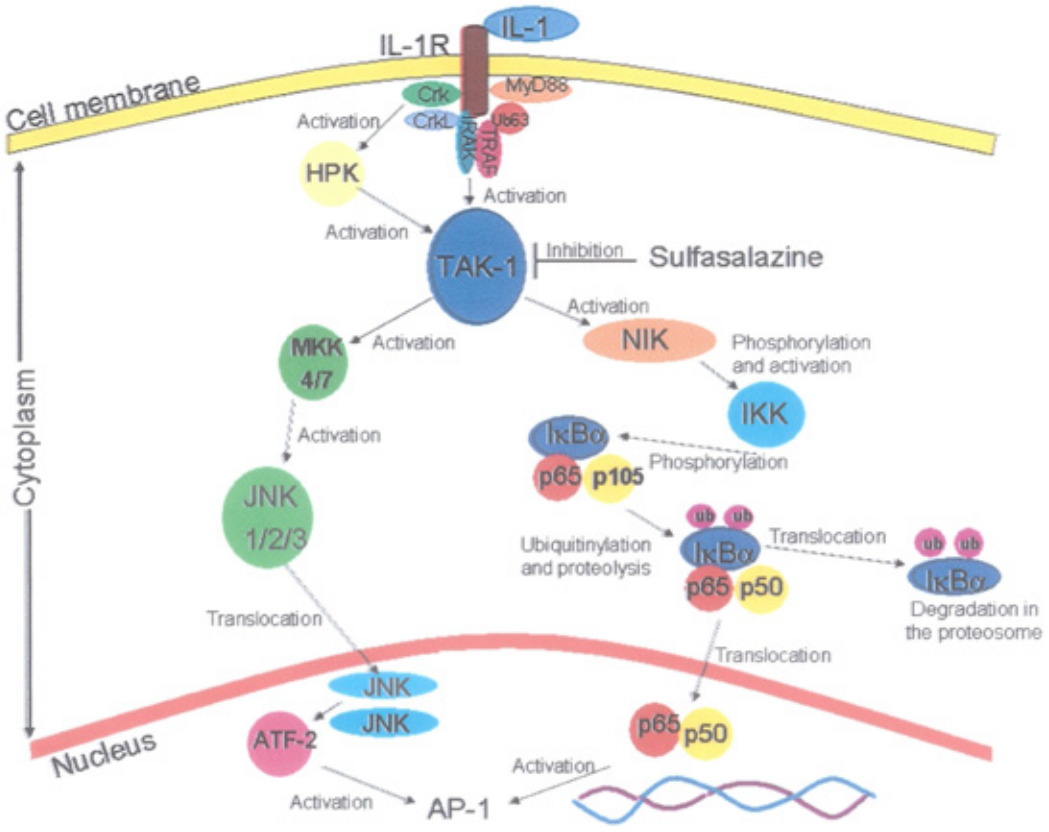
In this study it has been demonstrated that sulfasalazine is able block degradation of IkBα by inhibiting phosphorylation, thus supporting the documented evidence that sulfasalazine is a potent and specific inhibitor of NF-κB activation by blocking proteosome-mediated degradation of IκBα. A number of studies looking into possible effects by sulfasalazine on AP-1 in a variety of cell types have shared varying opinions. One paper suggested that whilst sulfasalazine clearly inhibits NF-κB activation in colon cells, AP-1 activity was not affected (Wahl et al, 1997). However another study suggested that the inhibition of Gadd45b by sulfasalazine in hepatic stellate cells may lead to the activation and phosphorylation of JNK being blocked and may ultimately lead to blocking AP-1 activity (Elsharkawy et al, 2005). In this study the PANARAMA signalling microarray was used as a method of screening the MAPK pathways for possible effects by sulfasalazine on IL-1 + OSM articular chondrocytes. Results obtained through the array highlighted the

possibility of sulfasalazine targeting a part of the MAPK pathway which goes on to activate AP-1 and western blots were carried out to confirm these findings. The western blots showed that sulfasalazine appeared to block the phosphorylation of MAPK kinase kinase 4 (MKK4) which is found high up in the JNK pathway. Phosphorylated MKK4 normally carries on to activate JNK 1/2/3 causing its phosphorylation and ultimately its translocation to the nucleus where it phosphorylates and activates ATF-2 causing the ATF to bind to AP-1 causing its activation. It seems that by blocking the phosphorylation of MKK4, the western blots showed that the events down stream of MKK4 also appeared to be blocked and thus preventing AP-1 activation.

From the results shown in this chapter it is indeed possible that sulfasalazine may be able to block the effect of cartilage degradation by targeting a number of cell signalling mechanisms namely NF-κB and for the first time JNK signalling with MKK4 and ATF2 which is shown to lead to AP-1 activation, both of which are known to be involved in the disease processes of arthritis and go on to cause the destruction of the articular joint. The results also suggest that sulfasalazine is likely to be inhibiting upstream of MKK4. TGFβ activating kinase is known to be found upstream of MKK4 in the MAPK pathway and can also be found upstream of IκB in the NF-κB pathway, so it may be possible that sulfasalazine is inhibiting both pathways by targeting TAK-1 and preventing it from activating downstream signals (Figure 7.9). With this in mind, there are a number of factors which need to be taken in to account, firstly the use of the Panorama signalling microarray for the screening of the pathways was indeed useful, however due to limited resources only one sample for each treatment could be analyzed and therefore the data shown was only representative of one sample. It is therefore necessary to be over cautious in interpreting too much from the results obtained, with this in mind the lack of effects seen with other antibodies on the array may not necessarily be a true result and further investigation of these pathways may be necessary. The quality of the antibodies used may also be a factor and with more time and a better quality of antibody, clearer and maybe more accurate results maybe seen. However, if the data shown is taken as being preliminary work, this line of investigation may potentially show a new mechanistic action by which sulfasalazine is able to block the destruction of cartilage matrix.

Figure 7.9 Inhibition of NF-κB and JNK signalling by sulfasalazine.

IL-1 receptor (IL-1R), TNF receptor-associated factor 6 (TRAF6), Myeloid differentiation primary response gene 88 (MyD88), IL-1R-associated kinase (IRAK), Ubiquitin 63 (Ub63), TGFβ activating kinase (TAK-1), NF-κB–inducing kinase (NIK), IκB kinase (IKK), κB inhibitor (IκB), Hematopoietic progenitor kinase 1(HPK 1), MAPK kinase kinase 4 (MKK4), c-Jun N-terminal kinase (JNK), Activating transcription factor-2 (ATF2), activator protein 1 (AP-1)



Summary

Results in this chapter have shown that:

- The passaging of human articular chondrocytes past passage 1 causes the cells to undergo phenotypical changes.
- Sulfasalazine is able to block proteosomal-mediated degradation of IκB and therefore inhibits NF-kB activation.
- Sulfasalazine appears to block the activation of AP-1 by targeting and preventing the phosphorylation of MAPK kinase kinase 4, JNK and ATF2.

CHAPTER 8: General Discussion

Arthritis is a severe and debilitating disease of the joint which can manifest itself in many ways. There is a vast array of arthritic diseases, with the most common types being rheumatoid arthritis and osteoarthritis. Rheumatoid arthritis is known to be a chronic connective tissue disease associated with bone and cartilage destruction. It is considered to be an inflammatory auto-immune disorder that causes the immune system to attack the joints. Known to affect more women than men, rheumatoid arthritis becomes more prevalent with age though its common age of onset is often between the ages of 40 and 50. Affecting about 1% of the British population, patients tend to suffer from pain, stiffness and swelling of the joints, which leads to the irreversible destruction of the joint and deformity (Lee et al, 2001). Studies investigating the disease process of rheumatoid arthritis have shown that the destruction of the rheumatic joint occurs when the pannus invades the superficial layers of joint cartilage (Woolley et al, 1997).

Osteoarthritis is a heterogeneous and multi-factorial disease caused by the loss of articular cartilage. It is commonly caused as a result of the patient suffering an alteration in mechanical loading and / or previous injury or trauma, with pain, stiffness and impaired movement being common symptoms. The most common form of arthritis, osteoarthritis can be found in any joint and is know to affect many people in the UK. The risk of developing the condition increases with age and mainly affects people over the age of 40 but it is most common among those over the age of 65. In fact, by the age of 65, around 70% of people have osteoarthritis in one or more of their joints, and around 10% have some disability caused by it. The burden of arthritis is indeed great and as people are progressively living longer this burden is only like to increase.

There are a number of different types of drugs which are used in the treatment of arthritis, including non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs). Whilst both types of drugs are used in the treatment of rheumatoid arthritis in order to relieve pain, inflammation and to slow disease progression, NSAIDs are the only form of treatment for osteoarthritis with the sole purpose of relieving pain. It is for this reason that patients with osteoarthritis eventually require a joint replacement operation. With this in mind the investigations carried out in this study bear great importance in not only preventing joint destruction in rheumatoid arthritis, but also

finding a possible therapeutic which can slow the disease progression in osteoarthritis and delay the need for joint replacement operations.

In this study, the use of therapeutics to prevent the destruction of cartilage collagen was investigated. It is well known that during rheumatoid arthritis the influx of cytokines into the joint, plays a key role in the destructive process. Cytokines such as IL-1 and OSM have been linked with joint destruction; with both being known to cause the super induction of inflammatory mediators such as the MMPs by cells within the joint (Pettipher et al, 1986; Langdon et al, 1997; Pelletier et al, 2003). During the process of joint destruction cartilage matrix is broken down caused by the irreversible destruction of collagen (Jubb and Fell, 1980). When IL-1 is used in combination with the OSM, a synergistic up regulation of matrix degrading enzymes leads to cartilage collagen breakdown. Data in this present study confirms the effect of using IL-1 in combination with OSM on collagen degradation. IL-1 and OSM are also known to up regulate the expression of the matrix degrading enzymes MMP-1, MMP-3 and MMP-13 (Koshy, 2002), which has also been confirmed by data presented in this study.

The importance of finding drugs which potentially may be used as treatment for osteoarthritis led to the screening of a number of drugs from various classes by the bovine nasal cartilage explant assay system in order to investigate their potential in inhibiting the release of cartilage collagen during the process of joint destruction. The NSAID indomethacin and its derivative indomethacin heptyl ester were selected due to their ability to inhibit COX-1 and COX-2 and previous studies had shown that indomethacin was able to inhibit MMP-1 in bovine articular chondrocytes (Sadowski and Steinmeyer, 2001). Sulfasalazine, a drug used in the treatment of early stage rheumatoid arthritis was selected to represent the disease modifying anti-rheumatic drugs (DMARDs), as whilst sulfasalazine is able to inhibit MMP-1, MMP-2 and MMP-3 in rheumatoid synovial fibroblasts (Minghetti et al, 2000), very little is known about its effect on blocking collagen release and its mechanisms of action. It was also decided that due to their apparent anti-inflammatory properties, statins should also be used to investigate possible chondroprotective effects. Hydrophobic statins, mevastatin and simvastatin were selected along with hydrophilic statin pravastatin, to see if they would block the release of collagen from cartilage, whilst also seeing if their structural differences had any effect.

In this study, a variation of results was seen with the NSAIDs. Indomethacin was seen to significantly inhibit the release of collagen from resorbing bovine nasal cartilage explants, and this was coupled with the down regulation of pro and active collagenases released into

the culture medium. However, this property was not seen with indomethacin heptyl ester which in fact caused the significant induction of collagen release. These findings lead to questions being asked about the differences in the specificity of the two drugs and indeed how their structural differences affect their ability to penetrate the cartilage explants, and thus inhibit cartilage breakdown. The outcome of the two drugs was further investigated using human articular chondrocytes, to look at how they affect the stimulated cells in monolayer. By looking at the consequence of the drugs on key cartilage degrading enzymes both transcriptionally and translationally it was possible to see that indomethacin did appear to block the release of collagenases from the cells with the significant reduction of the levels of both MMP-1 and MMP-13. As before treatment with indomethacin heptyl ester caused an increase in the expression of these enzymes, supporting the results seen in the release of collagen. The discovery of the indomethacin heptyl esters ability to induce the expression of the collagenases in monolayer suggest that it is not a case that structure of drug prevents it infiltrating the cartilage explants, but that in some way the structural changes made to indomethacin in order to form indomethacin heptyl ester have in someway caused it to become an inducing agent. Further investigations are therefore required to understand the differences in the outcomes seen.

In studying the consequence of the statins on cartilage breakdown a variation in results was again seen. With the addition of the statins to resorbing bovine cartilage explants a significant inhibition of collagen release was seen with both mevastatin and simvastatin, however there was no effect in reducing the release of collagen by pravastatin. The variation in these outcomes again suggests that possible structural differences between the drugs may affect their ability to infiltrate the cartilage matrix itself. Further investigation was again carried out using human articular chondrocytes in monolayer and by using the TaqMan low density array it was possible to investigate the consequence of the statins on the MMPs, TIMPs and ADAMTSs with pravastatin and simvastatin. The results obtained from this showed that both pravastatin and simvastatin were both able to inhibit the mRNA expression of the collagenase MMP-1, -8 and -13 in IL-1 + OSM stimulated chondrocytes. However results also showed that hydrophobic simvastatin caused an increase in the expression of MMP-3 whilst the hydrophilic pravastatin blocked MMP-3 expression. In order to investigate some of these results further the protein expression of MMP-1 and MMP-13 were measured by ELISA. The data showed the inhibition of MMP-1 by both pravastatin and simvastatin, whilst no effect was seen with mevastatin, however MMP-13 inhibition was seen with simvastatin and mevastatin, pravastatin caused the expression to

be increased. These data in this study strongly highlight the potential benefit of using statins in arthritis in order to block the degradation of cartilage within the joint. The use of both hydrophilic and hydrophobic drugs showed a variation of effects and therefore suggests that each drug works by a different manner of inhibition which requires further investigation. These outcomes are currently being investigated further within the department, using a broader spectrum of drugs and looking more in depth at the signalling mechanisms which maybe involved.

In this study the addition of sulfasalazine to resorbing bovine nasal cartilage explants resulted in the significant inhibition in the release of collagen and collagenase activity. A number of studies have looked at the ability of sulfasalazine to suppress the expression of MMPs from a number of different cell types. In a previous study using rheumatoid synovial fibroblasts, it was found that sulfasalazine was able to inhibit MMP-1, MMP-2 and MMP-3 (Minghetti et al, 2000), whilst another study using corneal fibroblasts found that although sulfasalazine inhibited MMP-1 and MMP-3 gene and protein expression, but only protein express of MMP-2 and MMP-9 were inhibited (Lu et al, 2006). Data from this study showed the inhibition of MMP-1, MMP-2, MMP-9 and MMP-13 at both a protein and gene level whilst, the gene expression levels of both MMP-3 and MMP-8, along with aggrecanases ADAMTS-1 and ADAMTS-4 were also found to be significantly down regulated, which not only supports previously published data but also shows some novel effects.

A number of pathways are known to be involved in the disease progression of arthritis due to their activation by IL-1. Sulfasalazine is well known to inhibit the activation of NF-κB, by blocking the proteosome-mediated degradation of IκBα and thus preventing the translocation of NF-κB to the nucleus (Wahl et al, 1998; Oakley et al, 2005). Data from this study supports this fact; however it is possible that sulfasalazine may in fact inhibit other pathways involved in the arthritic disease process. Data from this study demonstrated the possibility that sulfasalazine may also work by inhibiting the JNK MAPK pathway and ultimately preventing the activation of AP-1, by apparently blocking the phosphorylation of MAPK kinase kinase 4 (MKK4). In blocking the phosphorylation of MKK4 this prevents further signalling cascades which would eventually activate AP-1. However this data is novel and does require further investigation in order to confirm these findings, with the need to look higher up in the pathway itself to confirm which kinase

sulfasalazine targets. Further investigation of the MAPK pathway as a whole would also be advisable as this pathway is indeed complex and many overlaps can occur.

The investigations carried out in this study are of key significance for the future of arthritis treatments, with many of the drugs screened demonstrating potential chondroprotective properties by their ability to block the release of collagen from cartilage. These findings, coupled with the inhibition of cartilage degrading enzymes by the drugs suggest that a number of therapeutics may potentially be used to provide novel treatment for patients with not only rheumatoid arthritis but also for those with osteoarthritis in order to prevent the loss of cartilage during the progression of the disease. This could ultimately work to slow the progression of the cartilage matrix destruction, delaying the need for joint replacement operations in many of these patients and in some cases may prevent the need for these operations at all. However, the fact that a few of the drugs failed to inhibit the release of collagen from the cartilage explants, does suggest that further investigation is require to discover why it is that some drugs inhibit whilst similar drugs do not. The effects seen with the drug indomethacin heptyl ester also highlights that care must be taken when developing new drugs or derivatives of current therapeutics as the use of indomethacin heptyl ester as a treatment for arthritis could have drastic results by accelerating the disease process.

Having completed this investigation it has highlighted a number of changes which could be made. In some of the experiments the cytokine concentrations used may have been too high and therefore driving the system too hard. The use of low cytokine concentrations may in fact show a greater inhibition by the drugs. Having said this, the use of IL-1 in combination with OSM may also have complicated matters as far as investigating the signalling mechanisms is concerned, and therefore future work would benefit by using IL-1 on its own. There still remains the need for the further investigation of many aspects of this study, with perhaps an understanding of the differences in the drug structure being a key factor. However, to conclude the data shown in this study, does provide hope that a therapy to prevent cartilage destruction may soon be in use for patients with arthritis, therefore providing a much better quality of life to many people throughout the world.

Future work:

- Investigation of the structural differences between indomethacin and indomethacin heptyl ester to completely understand the different effects on MMP levels observed with each drug. A project to investigate their specific mechanisms of action and if these different outcomes reflect blocking of separate signalling pathways.
- Investigation of the effects seen after treatment with statins is required in order to understand their exact mechanism of actions and looking at a range of different statins in order to completely understand their therapeutic potential.
- Further investigation of the MAPK pathway is required to confirm the effects seen with sulfasalazine on AP-1 activation.
- The measurement of MMPs in human synovial fluid from a cohort of patients treated with one or more of the studied drugs, in comparison with those without treatment would help to further confirm their effect on the expression of MMPs.
- Separate clinical trials of statins and sulfasalazine in OA.

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