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TOWARDS DEFINING A ROLE FOR ZOLEDRONATE AS A DISEASE-MODIFYING TREATMENT IN OSTEOARTHRITIS: AN IN VITRO STUDY OF THE EFFECTS OF ZOLEDRONATE ON CARTILAGE AND CHONDROCYTE PROTEOGLYCAN METABOLISM

**A thesis submitted to the University of London
for the degree of Doctor of Medicine**

By Gawun Jah-Hung Chung

**Institute of Orthopaedics & Musculoskeletal Science
University College London
2008**

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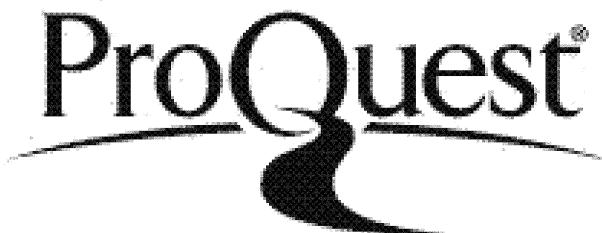


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DECLARATION

I declare that the work presented in this thesis is my own.

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Date: 13th April 2008

ABSTRACT

Osteoarthritis (OA) is a common group of disabling joint disorders for which there are limited pharmacological therapies to alter disease progression. Zoledronate, one of several bisphosphonates found to modulate joint changes in animal OA models, may have a disease-modifying role, and potential mechanisms of action include effects on cartilage and/or subchondral bone. In OA cartilage, loss of aggrecan, the main glycosaminoglycan-bearing proteoglycan, and degradation of type II collagen are major biochemical changes arising from imbalances in matrix synthesis and degradation. Zoledronate, in common with other bisphosphonates, is capable of inhibiting matrix metallo-proteinases, enzymes implicated in OA cartilage matrix catabolism, providing a biochemical basis for cartilage effects but it is not known whether direct effects occur at the cell/tissue level.

Studies described in this thesis have explored the hypothesis that zoledronate modifies cartilage metabolism to reduce cartilage glycosaminoglycan loss in OA. Short-term treatment effects on proteoglycan synthesis and degradation were examined *in vitro* in models of cartilage and chondrocyte metabolism, with IL-1 α used to stimulate “OA-like” tissue glycosaminoglycan release.

Zoledronate 10 $^{-4}$ M adversely affected cell viability, proliferation and proteoglycan synthesis in bovine articular chondrocytes and, thus, was the upper limit of the concentration range investigated. No enhancing effects were observed with zoledronate 10 $^{-10}$ M to 10 $^{-4}$ M on proteoglycan synthesis in bovine articular chondrocytes. No effects on glycosaminoglycan release were seen with zoledronate 10 $^{-10}$ M to 10 $^{-5}$ M in bovine articular cartilage or with zoledronate 10 $^{-8}$ M to 10 $^{-4}$ M in alginate bead constructs containing bovine articular chondrocytes and matrix.

Thus, a direct effect on cartilage proteoglycan metabolism following short-term treatment does not appear to be a mechanism of action for zoledronate as a disease-modifying treatment in OA. However, preventative or delayed treatment effects remain unaddressed and other potential targets for

zoledronate in the OA joint include cartilage type II collagen metabolism and subchondral bone metabolism.

ABBREVIATIONS AND CONVENTIONS

Conventions: International system (SI) units or units consistent with the recommendations of the International Committee for Weights and Measures are used throughout except that M is used to represent mol/l for expressing amount concentration.

List of abbreviations used in text:

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ACL	Anterior cruciate ligament
bFGF	Basic fibroblast growth factor
BMD	Bone mineral density
CDN	Canadian
CILP	Cartilage intermediary layer protein
COMP	Cartilage oligomeric protein
CV	Coefficient of variation
IC ₅₀	Concentration producing 50% inhibition
CTX-I	C-telopeptide of type I collagen
CTX-II	C-telopeptide of type II collagen
ECM	Extracellular matrix
Exp	Experiment
GAG	Glycosaminoglycan
IGF-1	Insulin-like growth factor-1
IGD	Interglobular domain
MMP	Matrix metalloproteinase
MT-MMP	Membrane-type MMP
OA	Osteoarthritis
rOA	Radiographic OA
SD	Standard deviation
srOA	Symptomatic and radiographic OA
SYSADOA	Symptomatic slow acting drugs for OA
TIMP	Tissue inhibitor of metalloproteinase
TGF- β	Transforming growth factor- β
UK	United Kingdom
US	United States
WOMAC	Western Ontario and McMaster Universities

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Chapter 1. Introduction

This chapter is arranged in three parts. The first part focuses on defining osteoarthritis and the relevance of the condition. Next, changes to synovial joints that are seen during the OA disease process are considered, along with underlying pathogenic mechanisms. In particular, alterations to articular cartilage and subchondral bone are covered, setting the scene for investigating treatments that might modify the course of the condition by targeting the metabolic processes in these two tissues. The last part reviews studies relevant to the concept of zoledronate as a disease-modifying treatment for osteoarthritis and ends with the direction for investigation.

1.1 Osteoarthritis: what is it and how is it relevant?

Osteoarthritis, which is also known as degenerative joint disease and osteoarthrosis, is the commonest disorder to affect joints and a major cause for locomotor disability. Though a fair amount of attention has been directed towards understanding OA, defining OA precisely has been problematic.

1.1.1 Definitions

Several definitions for OA have been put forward. In 1986, the Subcommittee on Osteoarthritis of the American College of Rheumatology Diagnostic and Therapeutic Criteria Committee proposed the following:

“OA is defined as a heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone and at the joint margins.” (Altman et al. 1986)

In that same year another definition was developed at a conference on the “Etiopathogenesis of Osteoarthritis” which summarised OA according to clinical, pathological, histological, biomechanical and biochemical characteristics:

“OA is a degenerative disease of the cartilage of joints. It is of diverse etiology and obscure pathogenesis. Clinically, the disease is characterized by joint pain, tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of local inflammation, but without systemic effects. Pathologically, the disease is characterized by irregularly distributed loss of cartilage more frequently in areas of increased load, sclerosis of subchondral bone, subchondral cysts, marginal osteophytes, increased metaphyseal blood flow, and variable synovial inflammation. Histologically, the disease is characterized early by fragmentation of the cartilage surface, cloning of chondrocytes, vertical clefts in the cartilage, variable crystal deposition, remodelling, and eventual violation of the tidemark by blood vessels. It is also characterized by evidence of repair, particularly in osteophytes, and later by total loss of cartilage, sclerosis, and focal osteonecrosis of the subchondral bone. Biomechanically, the disease is characterized by alteration of the tensile, compressive, and shear properties and hydraulic permeability of the cartilage, increased water, and excessive swelling. These cartilage changes are accompanied by increased stiffness of the subchondral bone. Biochemically, the disease is characterized by reduction in the proteoglycan concentration, possible alterations in the size and aggregation of proteoglycans, alteration in collagen fibril size and weave, and increased synthesis and degradation of matrix macromolecules. Therapeutically, the disease is characterized by a lack of a specific healing agent” (Mankin et al. 1986).

More recently in 1995 a further definition arose out of a workshop entitled “New Horizons in Osteoarthritis”:

“Osteoarthritis is a group of overlapping distinct diseases, which may have different etiologies but with similar biologic, morphologic, and clinical outcomes. The disease processes not only affect the articular cartilage, but involve the entire joint, including the subchondral bone, ligaments, capsule, synovial membrane, and periarticular muscles. Ultimately, the articular cartilage degenerates with fibrillation, fissure, ulceration, and full thickness loss of the joint surface.....OA diseases are a result of both mechanical and biologic events that destabilize the normal coupling of degradation and synthesis of articular cartilage chondrocytes and extracellular matrix, and subchondral bone. Although they may be initiated by multiple factors, including genetic, developmental, metabolic, and traumatic, OA diseases involve all of the tissues of the diarthrodial joint. Ultimately, OA diseases are manifested by morphologic, biochemical, molecular, and biomechanical changes of both cells and matrix which lead to a softening, fibrillation, ulceration, loss of articular cartilage, sclerosis and eburnation of subchondral bone, osteophytes, and subchondral cysts. When clinically evident, OA diseases are characterized by joint pain,

tenderness, limitation of movement, crepitus, occasional effusion, and variable degrees of inflammation without systemic effects." (Kuettner and Goldberg 1995)

In terms of understanding OA, the strength of these definitions lies in their description of the pathological outcomes of the OA disease process. Clearly OA leads to articular cartilage loss, underlying bone changes and alterations to other joint tissues. However, it is evident from these definitions that the links from aetiology to pathology to clinical outcome remain poorly understood, and it is not clear how different joint tissues interact during disease pathogenesis. Furthermore, because OA is a heterogeneous group of disorders, it is apparent that an all-encompassing definition of OA will either be a non-specific one or a lengthy one, more representative of a description.

Several investigators have advocated classifying the condition into subtypes, for example according to (i) the localisation of disease (inter- as well as intra-articular) and the current balance of degradation and repair in the joint (Dieppe 1995); or (ii) an initial division into idiopathic and secondary OA, and then sub-classification of idiopathic OA according to site(s) of involvement and secondary OA by cause (Altman et al. 1986; Altman et al. 1990; Altman et al. 1991). The use of such classifications in research is likely to be a fruitful way of furthering understanding of OA. Investigation of subsets of OA may reveal distinct aetiological factors, pathogenic pathways, outcomes and responses to treatment (Dieppe 1995). Furthermore, because each subset should demonstrate more homogeneity than the collective group of OA conditions, the classification approach should allow clearer delineation of the path from aetiology to clinical outcome.

As more information becomes available about OA, and along with further analyses of the knowledge base, definitions will continue to evolve. Ultimately, in order to be clinically useful, a definition of OA should also provide insight into prognosis and likelihood of response to intervention.

1.1.2 Risk factors for OA

OA aetiology is multi-factorial with often complex links to pathological change in the tissues of the joint. In a comprehensive review by Felson, OA risk factors are grouped into those that are systemic, intrinsic, or extrinsic to the joint (Table 1.1) (Felson 2003).

Table 1.1. Risk factors for OA. Adapted from (Felson 2003).

Systemic factors	Intrinsic joint factors	Extrinsic factors acting on joints
Ageing	Joint site	Obesity
Female gender	Previous damage	Injurious activity
Oestrogen deficiency in women	Malalignment/ deformity	
Genetic susceptibility	Laxity/ instability	
Obesity	Muscle weakness	
Nutritional factors	Proprioceptive deficiency	
Bone mineral density*		

*The relationship between bone mineral density (BMD) and OA is complex; high BMD is associated with new onset OA but low BMD is associated with OA disease progression (Hart et al. 2002; Zhang et al. 2000)

In some cases a single over-riding risk factor can be identified, as in the case of anterior cruciate ligament instability and the development of knee OA (Sherman et al. 1988). More commonly there is interplay between risk factors such as the interaction of age and trauma: older age in subjects who sustain a knee injury with meniscal damage is associated with more rapid development of OA change (Roos et al. 1995). Complexity is additionally compounded by the fact that OA has a gradual onset during which several risk factors may act at different time periods. Furthermore the role played by a particular risk factor may vary according to the stage of the disease (Doherty 2001; Felson 2003) to the extent that, paradoxically, roles can be opposing at different times. In the case of bone mineral density (BMD) and OA, high BMD is associated with new onset OA whereas low BMD appears to be a risk factor for disease progression in patients that already have OA (Hart et al. 2002; Zhang et al. 2000).

1.1.3 Public health relevance

The importance of OA worldwide is reflected by global burden of disease estimates from the World Health Organization which indicate that in 2002 the condition was the eighth leading cause of non-fatal disability, with a disease burden similar to that for schizophrenia or age-related vision disorders (Beaglehole et al. 2004). In the future disease burden is likely to increase for a number of reasons. Firstly, the projected increases in the population aged 65 years and older (to approximately double by 2020 compared to 1990) (Murray and Lopez 1997) will lead to more cases of OA because of high disease prevalence in the elderly. Secondly, increasing rates of obesity, an important risk factor for developing OA, in societies such as the UK will elevate the disease burden. Furthermore, socio-economic drives to promote independence among older people will mean additional resources are needed in order to reduce the disability profile of OA.

1.1.3.1 Prevalence

OA has a predilection to target certain joints whilst sparing others. Commonly affected joints are the small joints of the hand (distal interphalangeal, proximal interphalangeal and the first carpometacarpal joints), the hip joint, the knee joint, the cervical spine and the lumbrosacral spine (Felson 2003). The basis for this distribution is not well understood, though it has been suggested that new biomechanical demands that have been placed on particular joints as humans have evolved are significant (Felson 2003). For example, full weight-bearing on the legs will have altered the load distribution through hip and knee joints which may predispose these joints to OA.

OA is an extremely common condition and is particularly prevalent for the hand and knee (Table 1.2). When interpreting reported OA prevalence rates it is important to bear in mind which method was used for case identification. Classically OA has been diagnosed by the presence of radiographic change, frequently according to the grading system described by Kellgren and Lawrence

in 1957 which is based on the severity of joint space narrowing (as an indirect measure of cartilage loss) and various bony changes (subchondral and marginal) (Kellgren and Lawrence 1957). Other classification methods combine patient-reported pain and/or other clinical criteria with radiographic features to diagnose OA, such as those proposed by the American College of Rheumatology Subcommittee on Osteoarthritis for hip OA (Altman et al. 1991) and knee OA (Altman et al. 1986). Though radiographic evidence for OA is clearly associated with joint pain (Davis et al. 1992; Felson et al. 1997; Lethbridge-Cejku et al. 1995; Spector et al. 1993) there is also a lack of overlap in many cases (Creamer and Hochberg 1997) and symptoms can be absent in about 90% of subjects with radiographic hand OA (Lawrence et al. 1966; Lawrence et al. 1998; Zhang et al. 2003) and up to about half of patients with radiographic knee OA (Dillon et al. 2006; Du et al. 2005; Felson et al. 1987; Jordan et al. 2007; Lawrence et al. 1966). Thus it follows that, for each particular joint region, application of combined clinical/radiographic classifications to a population will find substantially lower prevalence rates than the use of a classification based solely on the radiographic features. Furthermore, relying on patient-reported pain within a purely clinical diagnosis will miss asymptomatic cases with radiographic OA.

Based on the presence of clinical symptoms and radiographic criteria for OA, knee OA affects about 10% of the adult or elderly population (Table 1.2). Hand OA is generally less prevalent though reported rates are quite variable, ranging from 1.7% for adult men (Lawrence et al. 1998) to 26.2% for elderly women (Zhang et al. 2002), and the hip is the least commonly affected joint of the three with prevalence rates of less than 1% (Lawrence et al. 1998). Other generalisations that are apparent are that OA is more common in the elderly

Table 1.2. OA prevalence: estimated rates of hand OA, hip OA and knee OA for several countries

Joint region	Study population		% prevalence						Reference
			Radiographic OA with or without symptoms			Radiographic OA with symptoms			
	Country	Age range (years)	Total	Female	Male	Total	Female	Male	
Hand	US	25-74	29.5	30.1	28.9	2.4	2.9	1.7	(Lawrence et al. 1998)
	US	≥40		69	31				(Wilder et al. 2006)
	US	71-100					26.2	13.4	(Zhang et al. 2002)
	UK	≥15		24.4	18.2		5.9	2	(Lawrence et al. 1966)
	China	≥60		47	44.5		5.8	3	(Zhang et al. 2003)
	Finland	≥30	44.8						(Haara et al. 2003)
Hip	US	55-74	3.2	2.8	3.5	0.7	0.7	0.7	(Lawrence et al. 1998)
	China	60-89		0.9	1.1				(Nevitt et al. 2002)
	Denmark	≥60	4.4-5.3						(Jacobsen et al. 2004)
Knee	US	≥45	16			8			(Jordan et al. 2007)
	US	≥60	37.4	42.1	31.2	12.1			(Dillon et al. 2006)
	US	63-94	33	34.4	30.9	9.5	11.4	6.8	(Felson et al. 1987)
	UK	≥35	18.4	22.6	14.2	9.4	12.4	6.4	(Lawrence et al. 1966)
	China	≥40	37.4			7.2			(Du et al. 2005)

US, United States; UK, United Kingdom

and, for the hand and knee, the condition more frequently affects women than men (Table 1.2).

1.1.3.2 Economic cost

Another way to consider public health relevance is according to the economic cost of OA. For individual patients, the direct cost of illness is the expenditure for medical care and the indirect cost arises from the impact of the illness on function, usually measured by wages lost but also can include implied losses from reduced home and leisure activities (Yelin 1998). The monetary cost felt by society is the total of these costs per disease case multiplied by the disease prevalence.

In a US community-based cohort, average direct costs over a one-year period for patients with a diagnosis of OA were found to be US\$2 654.51 compared to US\$1 387.83 in patients without arthritis after adjusting for age and sex (Gabriel et al. 1997). Another estimate of annual direct costs due to OA, through analysis of insurance claims, gave per patient costs of US\$5 294 and US\$5 704 for OA patients aged <65 and ≥ 65 years, respectively, and corresponding figures of US\$2 467 and US\$3 741 for age and sex matched controls (MacLean et al. 1998).

The total annual costs among Canadian individuals with disabling hip and knee OA were found to be US\$9 882 per patient with indirect costs accounting for 80% of the total (Gupta et al. 2005). For the US, total yearly costs for each case of OA have been estimated to range between US\$13 000 to US\$15 000 (Yelin 2003). Again indirect costs were more significant, at around US\$12 000.

In terms of regional population costs, a French study found evidence for the increasing impact of OA in financial terms (Le Pen et al. 2005). Direct medical costs attributable to OA in 2002 exceeded 1.6 billion Euros (US\$1.4 billion), making up 1.7% of the French health insurance system expense, and

represented a 156% increase compared to costs in 1993 (though adjustments for the effects of inflation were not specified in the study methodology). The increment was chiefly ascribed to 54% more OA patients with costs per OA patient only increasing at 2.5% per year.

An estimate of the total costs of OA for the US population has been derived from the analysis of the data from four studies published between 1984 and 1997 after adjusting amounts to 1999 US dollars (Yelin 2003). Based on the assumption that the population prevalence of OA was 4.2% for males and 9% for females, total costs for OA were calculated at US\$178.9 billion.

Costs bearable by society are also significant in the UK. Data from studies commissioned by the Arthritis Research Campaign indicate that in Great Britain 36 million working days were lost because of OA in 1999-2000 representing £3.2 billion (US\$2 billion) lost production (Arthritis Research Campaign 2002). Further more, OA accounted for 3 million GP consultations in 2000 and 114,628 hospital admissions in 1999-2000.

Overall the economic impact of OA on society appears to be substantial, whether per patient or for whole populations, with indirect costs being more significant than direct costs.

1.1.4 Clinical relevance

The primary clinical manifestations of OA are confined to the musculoskeletal system and, reflecting the heterogeneity of OA aetiology and pathogenesis, these features vary from case to case. Generally the clinical features are slow to evolve, lack an inflammatory aspect and usually only involve one or a few joints (although polyarticular OA is also common) (O'Reilly and Doherty 2003). The patient's perspective of OA can be considered in terms of problematic symptoms and functional disturbance.

Symptoms

The main symptoms that OA patients experience are pain and stiffness, with deformity being another complaint. The pain is usually aching in nature, related to joint use and alleviated by rest (O'Reilly and Doherty 2003). With progressive disease, the pain may become persistent, occurring at rest and at night. For individual patients, the overall perception of pain from OA joints is influenced by non-physical factors and studies of patients with knee or hip OA have found significant associations between measures of depressive and anxiety states and pain (Salaffi et al. 1991; Summers et al. 1988). Additionally, social circumstances and coping strategies are considered to be important determinants for pain (Creamer 2004; Hadler 2003).

As a symptom, "stiffness" of a joint can vary in meaning between patients, being used to describe difficulty or pain with joint movement. Early morning stiffness is generally short-lived compared to the prolonged stiffness that characterises inflammatory arthritis. Many OA patients complain of stiffness or "gelling" of joints after a period of inactivity to describe a transient difficulty in initiating movement of their affected joints.

How pathological changes to the joint structure in OA can lead to the pain experienced by the patient is not clear. As noted above, joint pain and radiographic evidence for OA are often discordant (Table 1.2). However, there is clearly an association between OA structural change seen on the X-ray and joint pain (Davis et al. 1992; Felson et al. 1997; Lethbridge-Cejku et al. 1995; Spector et al. 1993) and this relationship is more commonly observed in patients with a longer history and more persistent symptoms (Duncan et al. 2007).

No single specific articular source for pain in OA has been shown, though joint line tenderness elicited on clinical examination suggests a capsular or intracapsular origin (O'Reilly and Doherty 2003). Furthermore, transient abolition of pain has been achieved in 60% of patients following an intra-

articular injection of local anaesthetic into their OA knees (Creamer et al. 1996) and clear improvements in pain are seen after replacement of the diseased joint for knee or hip OA (Bachmeier et al. 2001; Escobar et al. 2007; Kirwan and Silman 1987).

Bone and surrounding periosteum, which are richly innervated with sensory nerve fibres (Mach et al. 2002), may be particular sources for pain. A cross-sectional observational study of patients with radiographic knee OA found that bone marrow lesions, seen on magnetic resonance imaging and thought to represent oedema, were more prevalent in subjects with knee pain (77.5%) compared to those with no knee pain (30%) (Felson et al. 2001). Because cartilage is aneural, it cannot be the originator for nociception, but an indirect role is suggested by the finding that chondral defects scored on MRI imaging were associated with knee pain in a dose dependent fashion (Zhai et al. 2007). Potentially, pain may arise from other joint tissues that are innervated such as synovium, capsule or soft tissue. Mechanisms beyond specific joint tissues may be also important and it has been proposed that alterations to peripheral and central nociceptive pathways in OA subjects increase the sensitivity of the joint to usually non-noxious stimuli (e.g. standing and walking), thereby contributing to the experience of pain (Kidd 2003).

Other clinical features of OA are more readily explained by structural change. Deformity and instability can arise from cartilage loss, bony deformity (through subchondral bone remodelling and osteophyte formation), ligament damage or laxity and muscle weakness. Reduced range of movement in a joint principally results from bone deformity and capsular thickening and is accentuated by effusion and soft tissue swelling (O'Reilly and Doherty 2003).

Functional impairment

Disabilities arising from OA include impaired mobility, problems with the activities of daily living, other restrictions in usual physical function and social difficulties such as lost work opportunities. Restrictions in mobility appear to

have particularly significant implications for the elderly, as illustrated by the finding in a Swedish cohort that walking speed at age 70 years predicted independence at age 76 (Sonn 1996). The mechanism for disability in OA is not always clear but painful joints, muscle weakness and reduced range of joint movement have been considered as contributors (O'Reilly and Doherty 2003). Other extra-articular factors also appear important in determining disability outcomes. For individuals with knee OA, age and body mass index were the non-local factors that were found to increase the risk of a poor functional outcome whereas mental health, self-efficacy, social support and aerobic exercise protected against disability (Sharma et al. 2003).

1.1.5 Management and disease modification

Because the clinical manifestations of OA have a heterogeneous and multifactorial nature, the management of patients with OA requires tailoring for the individual according to local joint features and, in addition, psycho-social factors. The aims of management are to educate the patient, control pain, optimise function, reduce handicap and beneficially modify the OA process (Brandt et al. 2003b).

The American College of Rheumatology, the European League Against Rheumatism and the National Institute of Clinical Excellence (UK) have published guidelines covering the management of hand, hip or knee OA which have recommended therapies based on the analyses of published evidence combined with expert opinion (2000; Jordan et al. 2003; National Collaborating Centre for Chronic Conditions 2008; Pendleton et al. 2000; Zhang et al. 2005; Zhang et al. 2007). Interventions that were identified as being beneficial for improving symptoms or functional outcome included non-pharmacological treatments (e.g. education, exercise, appliances and weight reduction), oral and topical analgesics, symptomatic slow acting drugs for OA (SYSADOA; e.g. glucosamine sulphate, chondroitin sulphate, diacerhein, avocado soybean

unsaponifiable and hyaluronic acid), intra-articular steroids and surgery (e.g. osteotomy and joint replacement).

Recently, attention has been directed towards the concept of structure- or disease-modifying drug treatments that might be able to slow the progression of OA and/or promote reparative processes (Abramson et al. 2006; Altman 2005; Sun et al. 2007). Findings from clinical trials have suggested disease modifying properties for doxycycline (Brandt et al. 2005), diacerhein (Dougados et al. 2001) and glucosamine sulphate (Bruyere et al. 2004; Pavelka et al. 2002; Reginster et al. 2001). In addition, there is some evidence for OA disease modification with intra-articular hyaluronic acid therapy (Goldberg and Buckwalter 2005). Proposed mechanisms of action vary between these agents and include effects on cartilage that may reduce matrix degradation or promote tissue repair (Table 1.3). However, despite the promising findings for these agents, drug treatments with indications for altering the OA disease process have yet to become established in clinical management guidelines and, currently, this overall approach remains largely conceptual.

Table 1.3. Possible OA disease-modifying agents and their mechanisms of action.

Possible OA disease-modifying agent	Possible mechanisms of action	References
Doxycycline	Inhibition of collagenase-mediated degradation of cartilage collagen	(Smith, Jr. et al. 1996; Yu, Jr. et al. 1991)
Diacerhein	Inhibition of IL-1 or MMP-mediated cartilage degradation	(Boittin et al. 1993; Martel-Pelletier et al. 1998; Moore et al. 1998)
Glucosamine sulphate	Stimulation of cartilage proteoglycan synthesis or inhibition of aggrecanase-mediated degradation of cartilage aggrecan	(Bassleer et al. 1998; Sandy et al. 1998)
Hyaluronic acid	Viscosupplementation of synovial fluid, promotion of cartilage repair, or inhibition of cartilage degradation	Reviewed in (Goldberg and Buckwalter 2005)

1.2 Osteoarthritis pathogenesis: targets for disease modification

Delineation of OA pathogenic pathways will aid identification of targets for disease modification. In the following sections, a conceptual framework for considering OA aetio-pathogenesis is described before turning to compositional and structural changes that affect articular cartilage and subchondral bone, two tissues that undergo characteristic changes in OA. In particular, the mechanisms driving these changes are explored, and considered as potential targets for modifying the OA disease process. Throughout these sections, a recurring theme is the intimate relationship between composition, structure and physical function.

1.2.1 The synovial joint: composition and function

The synovial joint is the commonest type of articulation in the human body and, like any joint, forms a junction between two or more bones of the skeleton. As integral parts of the musculoskeletal system, synovial joints participate in providing stable support structures that are able to distribute load and allow purposeful movement of different parts of the body relative to each other. Seven tissues make up synovial joints: articular cartilage, subchondral bone, synovium, synovial fluid, fibrous capsule/ ligament, tendon and muscle (Fig. 1.1). Each tissue has individual properties and together they complement each other to fulfil joint function.

1.2.2 OA pathogenesis: a dynamic process involving the whole joint

Views of OA pathogenesis have evolved. Previously OA has been seen as a passive degenerative “wear and tear” process leading to articular cartilage loss and subchondral bone change. More recent concepts recognise that OA is a process involving the entire joint during which important interactions occur between the different joint tissues (Brandt et al. 2003a), though there are competing theories as to which joint tissue change is of primary importance (Brandt et al. 2006).

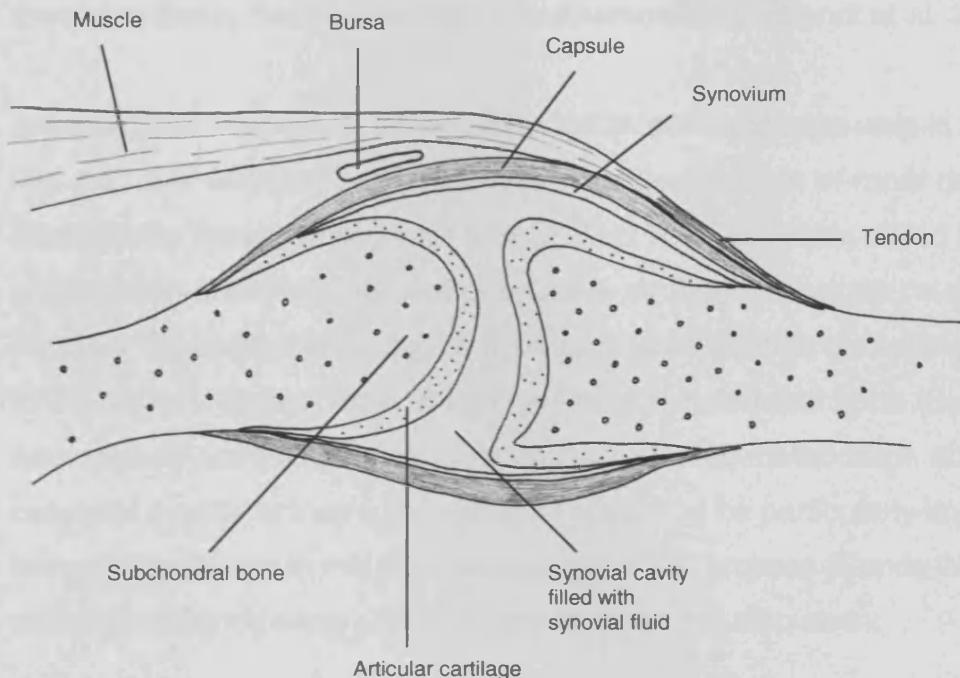


Fig. 1.1. Schematic representation of a synovial joint.

Articular cartilage: thin firm visco-elastic layer of connective tissue that forms a smooth articulating surface covering the articulating ends of bones.

Subchondral bone: cortical and cancellous bone layer that physically protects and supports the overlying cartilage.

Synovium: thin non-adherent soft connective tissue layer lining the cavity not covered by cartilage that secretes hyaluronan, a large molecular weight carbohydrate, into the joint cavity.

Synovial fluid: Hyaluronan-rich fluid filling the synovial cavity that acts as a lubricant for cartilage surfaces.

Capsule: basket work of strong dense connective tissue (ligament and tendon) around a joint that provides stability to the structure. Ligaments also hold bones together and restrain movement in particular directions. Tendons also connect muscles to bone.

Muscles: force-generating tissue attaching to bone via a tendon that is responsible for producing movement of joint structures relative to one another. They also assist in providing stability.

Bursa: structure lined with synovium and filled with synovial fluid that facilitates movement between different tissue planes.

Furthermore, the development of OA is viewed to involve metabolically-mediated tissue destruction, repair and remodelling (Brandt et al. 2003a).

A conceptual framework for considering OA aetio-pathogenesis is shown in Fig. 1.2. The way that each joint tissue responds to one or more risk factors depends on the prevailing joint constitution. This response, called the OA process can include acute and destructive structural changes caused by physical forces sustained by the joint, such as fibrillation of cartilage (Pritzker 2003) and microcracking or microfracture of subchondral bone (Burr 2003). Alternatively, joint tissues can respond with altered metabolism, and in cartilage catabolic events at the molecular level appear to be particularly important for tissue degradation in the early stages of the OA process (Sandy 2003). On the other hand remodelling and attempts at repair are also seen.

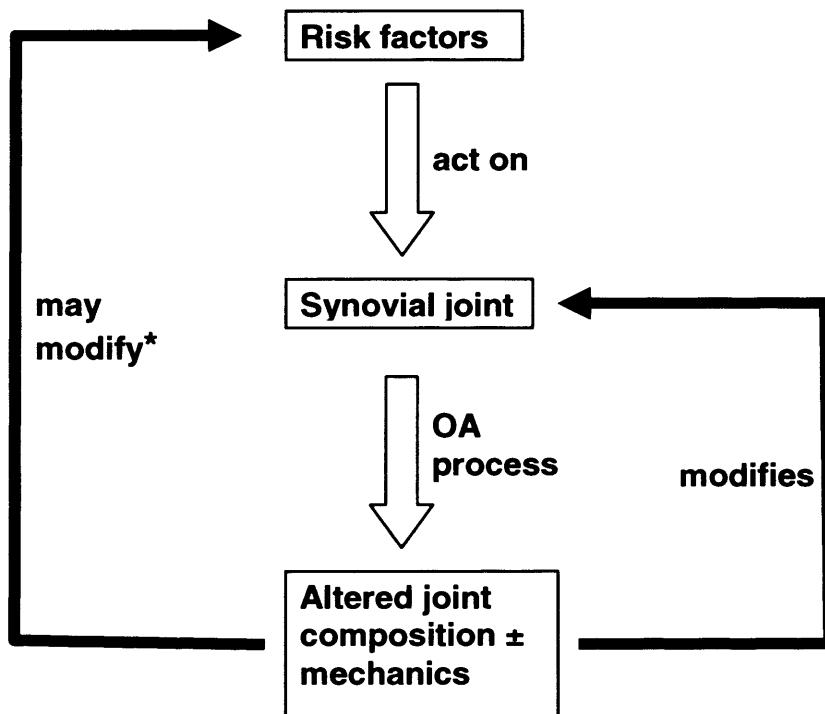


Fig. 1.2. OA aetio-pathogenesis as a dynamic process.

*Altered joint mechanics may modify bio-mechanical risk factors

The cells resident in cartilage proliferate (Mankin et al. 1971; Mankin and Lippiello 1970) and increase their synthetic activity (Adams and Brandt 1991; Mankin and Lippiello 1970; Nelson et al. 1998). In addition, new cartilage forms at joint margins where it ossifies into osteophytes (Brandt et al. 2003a; Moskowitz and Goldberg 1987) and subchondral bone undergoes remodelling (Burr 2003; Moskowitz 1999).

Changes to other joint tissues include joint effusions (with variable synovitis that is usually not intense), capsular thickening, ligament laxity or instability and muscle weakness (O'Reilly and Doherty 2003). The sum effect is a joint with altered composition, structure and attendant mechanical properties (Fig. 1.2). As changes develop, the new joint constitution then becomes the substrate for the influence of on-going or new risk factors. The effects of the risk factor older age illustrate this dynamic and continuous nature to the OA process. Older age is associated with a reduction in the tensile properties of human articular cartilage from the femoral head (Kempson 1991). This constitutional change exposes the aged cartilage to increased susceptibility to damage and, thus, trauma to the joint (another risk factor) that might normally have been innocuous may perpetuate the OA process. Interestingly, such a process provides an explanation for the observation noted above, that the risk factors older age and knee injury can interact during the development of knee OA (Roos et al. 1995).

Changes that alter joint biomechanics will modify biomechanical risk factors felt by the joint, and thus can influence OA pathogenesis. In addition, changes in one joint tissue may have direct effects on another tissue. In particular, cartilage and subchondral bone may physically or biochemically interact, and this concept is discussed further below (section 1.2.4).

At any particular point in time, the structural outcome of the OA process depends on the balance between the destructive/ degradative events and the effects of the repair response. The process can be slow and, depending on the

outcome measure, structural changes may take many years before they are evident. In patients who sustain anterior cruciate ligament or meniscal damage, the first signs of radiographic joint space narrowing appear, on average, ten years after injury (Roos et al. 1995).

Changes in articular cartilage and subchondral bone as targets for disease modification

Despite the under-explained link between OA structural change and symptoms, preventing or reversing structural change presents one approach to disease modification. Because OA aetio-pathogenesis is multi-factorial and leads to heterogeneous outcomes that can involve all tissues of the joint, the scope for selecting targets for disease modification is wide. Broadly, there are two areas for investigation: therapies to target risk factors or to modify the pathogenic response in joint tissues. It is the latter area that is subsequently further considered in relation to cartilage and bone, two tissues of the joint that undergo characteristic structural and compositional change during the OA process. The following two sections will outline these changes and the underlying metabolic mechanisms and it is these mechanisms that are considered to present pharmaceutical targets for disease modification.

1.2.3 Articular cartilage changes in OA

1.2.3.1 Articular cartilage composition and function

Anatomical location and physiological function

Adult articular cartilage is a relatively thin layer of tissue that covers the articulating ends of bones in the synovial joint (Fig. 1.1). This layer of cartilage, usually of the hyaline type, is a firm but compliant viscoelastic connective tissue. The functions of articular cartilage are (i) to provide an interface between subchondral bone and the synovial space; (ii) to manage, transmit and withstand repetitive physical forces, particularly during joint loading; and (iii) to

form a smooth articulating surface with the opposite articulating surface, assisted by synovial fluid.

Histological architecture

Cartilage is composed of cells embedded in an extracellular matrix (ECM). On light microscopy, normal articular cartilage can be distinguished into several zones arranged in layers parallel to the joint surface. Differences in cells and ECM composition occur between these zones. Moving from the surface downwards, these zones are the superficial zone, the middle zone, the deep zone then the distinct tide mark that separates the deep zone from the calcified zone (Thonar et al. 1999). In addition, domains of ECM are described according to their proximity to the cell, namely pericellular, territorial and inter-territorial compartments (Kuettner and Thonar 1998).

Chondrocytes

Chondrocytes are the sole cellular elements of cartilage. They are sparsely distributed, making up 2-3% of the total tissue volume, and assume flattened disc shapes in the superficial zone with their long axes parallel to the articular surface, becoming ellipsoid or spherical in shape through middle and deep zones (Thonar et al. 1999). Chondrocytes are responsible for synthesising and regulating the ECM, processes that are regulated by biochemical and mechanical factors (subsequently discussed in section 1.2.3.6). Progenitor-like characteristics have been identified in a subpopulation of superficial zone chondrocytes (Dowthwaite et al. 2004) suggesting that cells in this zone are particularly important for cartilage growth and, possibly, repair.

Matrix composition

The physical properties of cartilage, vital for the management of the physical stresses and strains of the tissue, are intimately dependent on the biochemical composition and structural integrity of the ECM. Articular cartilage is a highly hydrated tissue, with water making up over two-thirds of the tissue weight (Bollet and Nance 1966; Venn and Maroudas 1977). The main structural

component of the matrix is type II collagen which is present as a three-dimensional network of fibrils that binds and enmeshes large aggregates of negatively charged proteoglycans (Heinegård et al. 2003). Other collagens and non-collagenous proteins are found in smaller amounts. The composition of normal articular cartilage is not static but undergoes continuous turnover. In adult cartilage, the half-lives of collagen and intact aggrecan are estimated to be in the order of 100 years and less than 5 years respectively (Maroudas et al. 1992; Maroudas et al. 1998).

Type II collagen

Ninety percent of collagen in adult cartilage is of the type II variety (Thonar et al. 1999). Type II collagen molecules are fibril forming and have the typical collagen structure of a long, stiff, triple stranded helix (Alberts et al. 2002). Synthesis occurs in chondrocytes with each collagen molecule being made up of three α polypeptide chains tightly wound around one another. The structure is stabilised by inter-chain hydrogen bonding between hydroxyproline and hydroxylysine and the triple helix is protected from proteolysis by the side chain constituents of their amino acid residues. Collagen molecules assemble with one another into fibrils where covalent cross-linking between lysine residues of the molecules is important for fibril stability and tensile strength. A small amount of type XI collagen is present within the fibril which may have a role in determining fibril thickness. Another collagen, type IX, is bound on the fibril surface and is thought to be involved in regulating fibril assembly (Heinegård et al. 2003).

Throughout articular cartilage, the collagen fibrils that make up the network vary in thickness. The thinnest fibrils occur in the territorial matrix close to chondrocytes within the superficial zone and, in general, fibrils become thicker with distance from cells and towards the deep zone. In addition, fibril orientation demonstrates anisotropy through articular cartilage in which, relative to the articular surface, fibrils are mostly parallel in the superficial layer, perpendicular

in the deep areas and variable in direction in the intermediate parts (Heinegård et al. 2003).

Type II collagen imparts tensile properties to cartilage and when tension is generated as cartilage is stretched, the strength and stiffness of the tissue is dependent on the integrity of the collagen fibrillar network (Mow and Hung 2003). Disruption of the network, such as by breaking down intermolecular cross-linking by treating cartilage with elastase, leads to loss of tissue tensile strength and stiffness (Bader et al. 1981).

Matrix composition: aggrecan

The second main extracellular macromolecule is aggrecan. This large aggregating proteoglycan has important hydrophilic properties and makes up approximately 90% of the mass of proteoglycans in articular cartilage (Thonar et al. 1999). It has the typical proteoglycan structure of a central core protein with multiple glycosaminoglycan side chains which comprise 90% of the macromolecular mass (Thonar et al. 1999). Synthesis occurs in chondrocytes prior to secretion into the matrix (Hardingham 2004). The core protein of aggrecan has several distinct functional domains:

- a) CS1 and CS2 domains that are heavily decorated with approximately 100 glycosaminoglycan chains, each being composed of repeating chondroitin sulphate units. Chondroitin sulphate is a disaccharide with two negatively charged groups: a sulphate group on the N-acetyl galactosamine moiety; and a carboxylate group on the glucuronic acid. As each chondroitin sulphate chain is made up of 40 to 50 disaccharide units the CS1 and CS2 domains contribute about 8000 to 10000 negatively charged groups to a single aggrecan molecule (Heinegård et al. 2003).
- b) N-terminal G1 domain that, with link protein, binds aggrecan to hyaluronan (Heinegård et al. 2003). Linkage of a large number of aggrecan molecules to hyaluronan, which is a long-chained

glycosaminoglycan, forms huge polymeric complexes that can be as big as a bacterium (Alberts et al. 2002). The large size of such aggregates is believed to aid its retention within cartilage.

- c) Other domains with less certain functions- a domain containing keratan sulphate glycosaminoglycan chains; a G2 domain that has homology with the G1 domain; and a C-terminal G3 domain with sequence homology to epidermal growth factor, complement regulatory protein and lectin (Heinegård et al. 2003). The G3 domain is important for secretion of aggrecan from the cell (Zheng et al. 1998).

Aggrecan that is retained within the collagen network contributes to tissue viscoelasticity, a behaviour of cartilage that is largely dependent on the ease of interstitial fluid flow through the matrix (Mow et al. 1980). Glycosaminoglycan chains of aggrecan form porous hydrated gels (Alberts et al. 2002) and fluid movement is governed by the pore size of these gels (Mow and Hung 2003). A further property of aggrecan that influences fluid flow comes from the negatively charged groups of the molecule. These impart a high fixed charge density that attracts "mobile" osmotically-active counter ions to balance electroneutrality, thereby generating an osmotic pressure that causes cartilage to imbibe and retain water. The resulting swelling pressure is met and balanced by tension in the restraining collagen network, a process that enables cartilage to reversibly deform during loading (Mow and Hung 2003).

Factors contributing to a reduced fixed charge density in cartilage, such as increased hydration or a lower glycosaminoglycan content, result in a tissue that is more compliant in compression (Mow and Hung 2003). For example, in-vitro digestion of proteoglycan in human articular cartilage by treatment with cathepsin D and cathepsin B has been shown to reduce viscous damping to compressive loads (Bader et al. 1992).

Matrix composition: other collagens and non-collagenous proteins

Several other types of molecules are found in small amounts in cartilage matrix. Type VI collagen may protect the chondrocyte from compression. Numerous non-collagenous proteins with less well understood functions have been also identified, such as the family of leucine-rich repeat proteins (COMP, cartilage oligomeric matrix protein; CILP, cartilage intermediary layer protein; fibronectin; perlecan; and matrilins) (Heinegård et al. 2003).

1.2.3.2 Cartilage metabolism during the OA process

A cardinal pathological feature of OA is articular cartilage loss. Tissue degradation occurs more frequently in areas of increased load (Mankin et al. 1986) and, macroscopically, this is first evident as fibrillation of the cartilage surface. Later on cartilage becomes eroded and this process can progress to full thickness loss of tissue that exposes subchondral bone (Pritzker 2003). In advanced OA joints, such as those that are excised at arthroplasty, though the cartilage loss is often extensive, some areas of cartilage can appear macroscopically normal (Brocklehurst et al. 1984).

At the molecular level, the loss of cartilage matrix during the OA process is driven by cell-mediated metabolic events. The principal ECM constituents, aggrecan and type II collagen, are altered both quantitatively and qualitatively through degradation and synthesis and ultimately it is the balance of catabolism and anabolism that is important for determining matrix composition (Nagase and Kashiwagi 2003). Thus, in order to alter compositional outcomes, the metabolism of aggrecan or type II collagen during the OA process present attractive targets for disease modification.

Other alterations that are seen in OA cartilage include chondrocyte hypertrophy, elevations in alkaline phosphatase activity and deposition of type X collagen in the matrix. In these respects, the tissue composition resembles immature cartilage (such as that found in the growth plate) which suggests that OA

cartilage is resuming its capability to mineralise (Doherty et al. 2004). Variations in the levels of matrix non-collagenous proteins (e.g. COMP, CILP and fibronectin) are also observed. Because these macromolecules are thought to have roles in matrix assembly, cross-linking networks and providing feedback to chondrocytes (Heinegård et al. 2003), these changes may be also important for the OA process.

1.2.3.3 Aggrecan metabolism in OA

Some of the initial changes in the OA process involve aggrecan molecules. Aggrecan is depleted from cartilage matrix thus removing fixed charge groups from the tissue with consequent alterations of physio-chemical properties (Heinegård et al. 2003). Though the loss of aggrecan from cartilage is considered to be reversible (Martel-Pelletier 2004; Mort and Billington 2001), it is thought to initiate the cascade of metabolic events leading to irreversible tissue disruption (Sandy 2006).

Early OA- cartilage proteoglycan content

Following joint injury and during the development of OA the degradation of aggrecan is evidenced by increased levels of fragments of this proteoglycan in the synovial fluid of human knee joints (Lohmander et al. 1999). Furthermore, with worsening arthroscopic and radiographic severity of OA there is an associated rise in the aggrecan fragment concentration of synovial fluid. In the cartilage tissue itself, aggrecan loss is reflected in pronounced reductions in proteoglycan staining (Heinegård et al. 2003). In addition, in dogs with knee joint instability following surgical transection of the cruciate ligament, the extent of this proteoglycan depletion increases with the degree of cartilage surface disruption (Visco et al. 1993).

Though these observations of reduced staining indicate a lower proteoglycan concentration in the tissue, in animal models of early OA the total proteoglycan content appears to increase (Adams et al. 1987; McDevitt and Muir 1976).

Similar findings are seen in human subjects who have sustained anterior cruciate ligament rupture; analyses of their femoral condylar cartilage reveals reduced histological staining for proteoglycan in some patients and an increase in tissue glycosaminoglycan content up to one year post injury (Nelson et al. 2006). These findings may be explained by cartilage swelling, an event that is also observed in early OA (Heinegård et al. 2003), whereby though tissue proteoglycan concentration may be reduced, the larger cartilage volume results in an overall increase in tissue proteoglycan content.

Late OA- cartilage proteoglycan content

Cartilage proteoglycan content is reduced in late-stage OA. By the time patients with OA undergo arthroplasty the disease can be considered to be advanced. Cartilage samples obtained from the knee or hip joints of such patients shows a decreased GAG content (Brocklehurst et al. 1984; Byers et al. 1977; Mankin and Lippiello 1970). In addition, the proteoglycan content of late-stage OA cartilage has been found to inversely correlate with OA severity at the hip joint as assessed histologically (Mankin et al. 1971).

Qualitative changes to aggrecan in OA

Apart from quantitative changes to proteoglycan in OA cartilage, qualitative alterations to aggrecan molecules are also seen. In OA cartilage, the chondroitin-4 sulphate: chondroitin-6 sulphate and chondroitin sulphate-rich proteoglycan: keratan sulphate-rich proteoglycan ratios have been observed to be higher than normal, resembling patterns seen in immature cartilage (Adams et al. 1987; Doherty et al. 2004). In addition, human OA cartilage from the femoral condyle has been found to bear elevated levels of a chondroitin sulphate epitope that is usually present in foetal aggrecan (Rizkalla et al. 1992). The implications of these biochemical changes are unknown, though some of the alterations may modify the binding of growth factors in matrix (Doherty et al. 2004). Another qualitative change is the presence of aggrecan fragments with shortened core proteins in OA cartilage (Sandy and Verscharen 2001). This finding indicates proteolytic cleavage activity, an important mechanism for the

release of GAG from the tissue that provides an explanation for the reduced GAG content in OA cartilage.

Mechanisms leading to the reduced GAG content in OA cartilage

Overall the findings in synovial fluid and cartilage in early OA indicate an increase in proteoglycan turnover, with increases in degradation and release from the tissue being accompanied by adequate synthesis to prevent any overall reduction in proteoglycan content. However, as the disease becomes more advanced the balance shifts and there appears to be a net loss of GAG from the tissue.

Mechanisms responsible for GAG release

a) Aggrecan core protein cleavage.

Aggrecan is present in cartilage matrix from normal, acutely injured and OA joints in various forms: the full-length species and a series of products generated by varying degrees of proteolytic truncation from the C-terminal end of the core protein (Sandy and Verscharen 2001).

Cleavage within the IGD releases the complete GAG-rich region from hyaluronan-bound aggrecan molecules leading to loss of GAG from the tissue. Thus, the IGD of the aggrecan core protein has been a major focus of investigation. Nine different cleavages within the IGD have been demonstrated to occur in incubations of aggrecan with purified proteinases but only two cleavages of the IGD appear to occur in human tissues *in vivo*: at the matrix metalloproteinase site and aggrecanase site (named after the principal enzyme families with activity at those sites) (Sandy 2006). Cleavage at these sites generates novel terminals (called neoepitopes) with specific N- or C- amino acid sequences that can be identified using antibody techniques (Caterson et al. 2000).

Are matrix metalloproteinases or aggrecanases responsible for aggrecanolysis in OA?

Matrix metalloproteinases (MMPs) and aggrecanases are thought to be the main proteinases involved in aggrecan degradation though the their relative contributions to aggrecanolysis in OA have recently been the subject of debate (Sandy 2006; Struglics et al. 2006). Both families of enzymes are present OA cartilage (Billinghurst et al. 1997; Curtis et al. 2002). In humans, both MMP and aggrecanase-generated cleavage products, identified either as neoepitopes or by protein sequencing, have been found in normal cartilage (Flannery et al. 1992; Lark et al. 1997), OA cartilage (Lark et al. 1997) and synovial fluid from OA joints (Fosang et al. 1996; Lohmander et al. 1993b; Sandy et al. 1992). One working model, based on analyses of human cartilage and synovial fluid from normal, injured and late-stage OA joints (Sandy and Verscharen 2001), proposes that aggrecanase activity is responsible for cleaving full length aggrecan and the loss of osmotically-active GAG. In this model, aggrecanase activity is thus considered “destructive” to the tissue. In contrast, MMP activity acts on a separate pool of aggrecan that does not bear much GAG and is therefore considered not to be “destructive” (Sandy 2006). Others have reported that synovial fluid from OA joints contains GAG-bearing aggrecan fragments generated by the activities of both MMPs and aggrecanases implicating both families of proteinases in aggrecan degradation in human OA (Struglics et al. 2006). In vitro studies of cartilage degradation find clear evidence for aggrecanase-mediated catabolism of aggrecan (Little et al. 1999) but also demonstrate that aggrecan cleavage involves MMP activity (Little et al. 2002).

b) Impaired aggrecan aggregation and altered hyaluronic acid metabolism.

Defective aggregation of aggrecan is a further possible mechanism for GAG release from cartilage in OA. The capacity of chondrocytes from ageing subjects to synthesize link protein and to assemble a

proteoglycan-rich matrix is impaired (Sandy et al. 1987). These impairments could lead to poor stabilisation of hyaluronic acid-aggregcan aggregates and loss of aggregcan from the tissue.

In addition, link protein-stabilised aggregates in vitro are less susceptible to hyaluronic acid degradation by hyaluronidase or free radicals (Rodriguez and Roughley 2006) so decreases in link protein synthesis with ageing may expose this vulnerability. Other in vitro studies have demonstrated the release of link protein and hyaluronic acid from bovine and human cartilage cultured in the presence of catabolic stimuli leading to the hypothesis that cleavage of hyaluronic acid and the release of small hyaluronic acid-aggregcan complexes is another mechanism for proteoglycan loss from the tissue (Sztrolovics et al. 2002).

Reversibility of GAG depletion from cartilage

A reparative ability of chondrocytes to respond to decreases in matrix GAG content is suggested by the observation that GAG synthesis inversely correlates with GAG content in human femoral head cartilage (Mankin and Lippiello 1970). Elevated GAG synthesis rates have been directly demonstrated in a rabbit model with instability-induced OA (Ehrlich et al. 1975). However, in the late stages of OA the synthetic response appears to reverse. Humans studies have shown reduced rates of GAG synthesis in cartilage from OA hip joints (Byers et al. 1977) and OA knee joints removed at arthroplasty (Brocklehurst et al. 1984). In addition, though GAG synthesis was shown to positively correlate with histological severity for cartilage obtained from excised OA hip joints, beyond a certain severity grade GAG synthetic rates appeared to decline (Mankin and Lippiello 1970).

Recovery from GAG depletion has been demonstrated in bovine articular cartilage explants in vitro. Using the catabolic cytokine IL-1 to induce depletion of GAG, explants were observed to re-accumulate GAG over a three week period if they were allowed to recover in the absence of IL-1 (Williams et al.

2003). Similarly, in an animal model of OA, the loss of proteoglycans induced by an intra-articular injection of IL-1 in rabbits was seen to replace gradually over a three to four week period (Page Thomas et al. 1991). Measurements of cartilage GAG synthesis showed that after initially depressed rates, there was a compensatory increase in GAG synthesis. Interestingly, this restorative response was found to be impaired in older animals (Arner 1994), a finding that offers some explanation for the association between ageing and OA.

1.2.3.4 Type II collagen metabolism in OA

During the OA process, overt collagen release is thought to follow the onset of proteoglycan loss from cartilage matrix (Caterson et al. 2000; Ellis et al. 1994). Though collagen is less readily released from matrix, this type of tissue disruption is considered to represent the point of irreversible cartilage destruction (Cawston et al. 1998; Jubb and Fell 1980). Initial changes to type II collagen arising from the OA process are qualitative in nature.

Physical changes to cartilage in OA and their relation to collagen

One early identifiable event in the OA process is oedema and swelling of cartilage (Heinegård et al. 2003). These changes are thought to probably reflect a loosening of the restraining collagen fibre network resulting in further hydration of the enmeshed proteoglycans and consequent swelling of the tissue (Venn and Maroudas 1977). In an ovine model of OA, meniscectomy was found to lead to areas of thicker more hydrated articular cartilage that demonstrated abnormal collagen birefringence intensity suggesting disorganisation of the collagen network (Appleyard et al. 2003). Further support for the role of collagen disruption in tissue swelling comes from the finding that the swelling of OA cartilage from the femoral condyle strongly correlated with the amount of damage to collagen molecules (Bank et al. 2000).

Another physical change to OA cartilage is that the superficial tissue layer is weaker and less stiff compared to normal cartilage (Kempson et al. 1973). Such

impaired tensile properties of cartilage have been found to relate to reduced tissue collagen content (Kempson et al. 1973) and also to damaged collagen fibrils (Bader et al. 1981).

Damage to type II collagen and its loss from cartilage during the OA process

Mirroring studies into aggrecan catabolism, many investigators have employed antibody techniques to identify neoepitopes revealed on collagen molecules when they are degraded. The initial cleavage of type II collagen generates two fragments, one approximately one-quarter in length and the other three-quarters in length compared to the original molecule (Miller et al. 1976). The resultant novel terminals of the molecule can be identified as cleavage neoepitopes (Billinghurst et al. 1997). In addition, cleavage of triple helical collagen leads to unwinding of the α chains which exposes another identifiable neoepitope, a denaturation neoepitope (Dodge and Poole 1989).

Within hours after trauma, higher levels of type II collagen fragments are seen in the synovial fluid of acutely-injured knees (Lohmander et al. 2003). In addition, the cartilage itself, taken at the time of anterior cruciate ligament (ACL) reconstruction surgery from acutely-injured knees, shows increased levels of type II collagen denaturation and cleavage though no change to the total collagen content (Nelson et al. 2006).

As the OA process progresses, evidence for collagen damage persists. The synovial fluid from patients with knee OA demonstrates increased levels of type II collagen fragments (Lohmander et al. 2003) and elevated cleavage and denaturation neoepitopes have been found in cartilage from OA knee joints removed at arthroplasty (Billinghurst et al. 1997; Hollander et al. 1994).

The amounts of cleavage and denaturation of type II collagen correlate with the histological severity of OA cartilage (Konttinen et al. 2005) and, within the tissue, these neoepitopes are mainly located in the pericellular matrix and in the superficial layer of cartilage (Wu et al. 2002).

In the later stages of the OA process, the collagen content of cartilage tissue is reduced (Hollander et al. 1994) and cleavage of collagen molecules appears to be an important underlying mechanism. In addition to increased levels of type II collagen fragments seen in the synovial fluid of acutely injured and OA knees (Lohmander et al. 2003), the amounts of type II collagen cleavage or denaturation neoepitopes in cartilage have been found to inversely correlate with collagen content (Poole et al. 2003; Squires et al. 2003). Furthermore, OA cartilage explants that were cultured *in vitro* released more type II collagen cleavage neoepitopes into culture media than non-arthritic cartilage explants (Dahlberg et al. 2000).

Mechanisms of type II collagen cleavage

Proteinases from the MMP family are thought to be responsible for collagenolysis in cartilage (Mort and Billington 2001). These degradative enzymes are found in the synovial fluid of acutely-injured (Lohmander et al. 1993a; Walakovits et al. 1992) and OA joints (Clark et al. 1993; Lohmander et al. 1993a). They are expressed in OA synovial tissue (Firestein et al. 1991) and expressed and secreted by OA chondrocytes at increased levels (Reboul et al. 1996). Tissue inhibitors of MMPs (TIMPs) are deficient relative to MMPs in OA cartilage and this imbalance is also likely to play a role in accelerated matrix degradation (Dean et al. 1989). Synthetic inhibitors of MMPs have been found to significantly reduce the release of type II collagen cleavage neoepitopes from OA cartilage cultured *in vitro* (Billinghurst et al. 1997; Dahlberg et al. 2000), clearly demonstrating the activity of MMPs in OA cartilage.

Synthesis as a repair response

During the OA process, increases in collagen synthesis suggest an attempt at repair. Knee joint instability, in a rabbit model of OA, has been found to stimulate collagen synthesis (Floman et al. 1980) and surgically-induced ACL rupture in canine knee joints increased cartilage type II collagen synthesis as measured by incorporation of [³H]proline into new collagen molecules (Eyre et al. 1980). Evidence of elevated synthesis of type II collagen is also found in

human OA cartilage. N- and C-propeptides are removed from type II procollagen fibrils as they are synthesised and secreted into ECM (Heinegård et al. 2003) and amounts of type II procollagen C-propeptide were found to be markedly elevated in human OA femoral condylar cartilage, particularly in the mid and deep zones (Nelson et al. 1998). Thus, there may be potential for repair of damage to the collagen network, but an effective response in late OA when major disruption in the collagen architecture has occurred is thought to be less likely (Doherty et al. 2004).

1.2.3.5 Cartilage tissue proteinases

ECM is metabolically degraded by various proteinases. During the OA process proteolytic enzymes are produced by chondrocytes and also synovial cells (Doherty et al. 2004). Table 1.4 lists proteinases that have been found in cartilage (Sandy 2003). Attention has focused on MMPs and aggrecanases as the proteinases responsible for the degradation of aggrecan and type II collagen in OA cartilage (Poole et al. 2003; Sandy 2006).

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of endopeptidases that have divalent cations as part of their active structure. They are secreted by cells found in joint tissues and are located in the matrix either as soluble proteins or bound to the cell membrane. Through their ability to degrade ECM constituents, MMPs are considered to play significant roles in normal physiological tissue remodelling, turnover and in pathological processes such as OA. In disease states, aberrant regulation of MMPs is thought to lead to their hyperactivity with consequent ECM degradation (Murphy et al. 2002).

The MMP family includes collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs). These proteinases have common domains in their protein structure with specific functions. The enzymes are secreted in

Table 1.4. Proteinases present in cartilage. From (Sandy 2003)

Enzyme family	Enzyme	Other name
MMPs	MMP-1	Collagenase 1
	MMP-2	Gelatinase A
	MMP-3	Stromelysin 1
	MMP-8	Collagenase 2
	MMP-9	Gelatinase B
	MMP-10	Stromelysin 2
	MMP-13	Collagenase 3
	MMP-14	MT1-MMP
ADAMTs	ADAMTS-1	METH-1
	ADAMTS-2	Procollagen-N-proteinase
	ADAMTS-3	Procollagen-N-proteinase
	ADAMTS-4	Aggrecanase-1
	ADAMTS-5	Aggrecanase-2
Others		
	Metallo-proteinase	Procollagen-C-endopeptidase
	Cysteine proteinases	Cathepsin B
	Serine proteinases	Calpain
	Aspartic proteinases	Plasmin
		BMP-1
		Plasminogen activator
		Cathepsin D

latent form and are usually activated extracellularly by cleavage of a propeptide domain. An N-terminal catalytic domain has a zinc ion bound at the catalytic site where hydrolysis is thought to occur by polarisation of a water molecule to act as a nucleophile to attack the scissile peptide bond. Further divalent cations, zinc and calcium, contained in the catalytic domain also seem significant for function. Cleavage of a substrate depends on the depth and structure of the catalytic site. Variations in this region between different MMPs are therefore important for the substrate specificity. A linker region connects the N-terminal catalytic domain to a C-terminal domain that has sequence similarity to the serum protein haemopexin. The role of this latter domain seems to vary

between MMPs but all collagenolytic enzymes appear to require this domain for the hydrolysis of triple-helical collagens (Murphy et al. 2002).

MMP-mediated cleavage of triple-helical collagens characteristically and specifically occurs at a single locus giving rise to fragments approximately three-quarters and one-quarter in length compared to the precursor molecule (Miller et al. 1976; Murphy et al. 2002). In articular cartilage, the initial cleavage of triple-helical type II collagen is thought to be mediated by MMPs-1, 8, 13 and 14 (Sandy 2003). The first cleavage then allows further degradation by other proteinases (Mort and Billington 2001). Selective inhibition of MMP-13 along with MMP-8 blocks the release of collagen from OA cartilage (Dahlberg et al. 2000). In particular, MMP-13 is considered to be important because it preferentially cleaves type II collagen (Knauper et al. 1996) and expression of human MMP-13 in the joints of transgenic mice was found to lead to OA cartilage changes (Neuhold et al. 2001). MMPs are also involved in aggrecan degradation as has been outlined above (section 1.2.4.1).

Other roles for MMPs include activation of other MMPs, for example MMP-14 (MT-1 MMP) is an activator of proMMP-2 on the cell surface (Murphy et al. 2002); release of matrix fragments that have cellular effects, for instance fibronectin fragments; release of growth factors or cytokines bound to the matrix or cell surface; and proteolytic activation or inactivation of growth factors, cytokines and their receptors (Murphy and Lee 2005).

Aggrecanases

The term “aggrecanase” refers to a proteinase that can cleave the aggrecan core protein at the glutamyl-X scissile bond which is insensitive to MMP activity (Sandy 2003). These glutamyl-X cleavage sites are located in the IGD and CS-2 domains of the aggrecan core protein (Caterson et al. 2000). Three members of the “A Disintegrin And Metalloproteinase with Thrombospondin motifs” (ADAMTS) family of enzymes have been identified as having aggrecanase activity: ADAMTS-1, ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) (Nagase and Kashiwagi 2003). Like MMPs, ADAMTSs also

have a metallo-proteinase domain that contains a zinc binding motif sequence at the catalytic site (Murphy and Lee 2005). Type-1 thrombospondin motifs are found in their structure and for aggrecanase-1 this motif is important for binding the enzymes to sulphated GAGs in the matrix and, thus, targeting of the proteinase to aggrecan molecules (Tortorella et al. 2000).

Other proteinases

Other proteinases implicated in cartilage breakdown are the cathepsin lysosomal proteinases (Mort and Billington 2001). In an inhibitor study, cathepsin B was implicated in IL-1-stimulated proteoglycan loss from bovine nasal cartilage (Buttle et al. 1993). Cathepsins have also been shown to degrade cartilage collagens (Maciewicz et al. 1990) and proteoglycans (Roughley 1977). However, these enzymes are considered to play a secondary role in cartilage degradation with mainly intracellular actions (Mort and Billington 2001). Furthermore, because their activity is optimum at acidic pH, it has been suggested that cathepsins might only participate in advanced stages of the OA process (Nagase and Kashiwagi 2003) when the local pH of cartilage has been shown to fall (Konttinen et al. 2002).

Tissue inhibitors metalloproteinases

Four mammalian tissue inhibitors of metalloproteinases (TIMPs) have been discovered. TIMP-3 is sequestered in the ECM and the other TIMPs localise to the cell surface. TIMPs-1 to 4 all inhibit MMP activity and TIMP-3 inhibits the aggrecan-degrading ADAMTSs-1, 4 and 5. Because the TIMPs are thought to have significant roles in regulating proteinase activity in ECM (Murphy and Lee 2005), the inhibitors are likely to be important for the maintenance of matrix integrity.

The mechanism of MMP inhibition by TIMPs involves the interaction of a “wedge-like” part of the TIMP protein and the active site of the MMP. This allows the amino and carbonyl groups of Cys1 residue at the N-terminal of the

TIMP molecule to chelate the catalytic zinc atom of the MMP, and expel the zinc-bound water molecule (Nagase et al. 2006).

1.2.3.6 Regulation of aggrecan and type II collagen metabolism

During the OA process, disturbance in the balance of anabolism and catabolism results in the net loss of cartilage matrix constituents and, therefore, leads to deterioration in the physio-chemical properties of the tissue (Goldring and Goldring 2004). Regulation of chondrocyte-mediated synthesis and degradation of ECM involves both biochemical and mechanical factors, both of which can interact.

Biochemical factors: cytokines

An extensive review on the role of cytokines in regulating cartilage degradation in OA has classified cytokines according to their catabolic or anabolic effects on chondrocyte-mediated matrix metabolism (Table 1.5) (Goldring and Goldring 2004). In addition, several cytokines have been described as anti-catabolic or modulatory based on their ability to inhibit or modulate the activities of other cytokines respectively. Within the tissue, cytokines act on cells by paracrine (cytokine produced by one cell acts on neighbouring cells) or autocrine (cytokine produced by a cell acts on the same cell) mechanisms (Goldring and Goldring 2004).

Although synovitis and/or clinical features of inflammation are not prominent in OA, proinflammatory cytokines derived from synovial tissues and chondrocytes are involved in cartilage destruction (Goldring 2000; Goldring and Goldring 2004). In particular, interleukin-1 (IL-1) and tumour necrosis factor- α (TNF- α) are considered to play major roles as catabolic cytokines (Martel-Pelletier et al. 1999).

Table 1.5. Classification of chondrocyte-cytokine interactions. From (Goldring and Goldring 2004).

Catabolic	Interleukin-1 Tumour necrosis factor- α Interleukin-17 Interleukin-18 Oncostatin M
Modulatory	Interleukin-6 Interleukin-11 Leukaemia inhibitory factor
Anti-catabolic	Interleukin-4 Interleukin-10 Interleukin-13 Interleukin-1 receptor antagonist Interferon- γ
Anabolic	Insulin-like growth factor-1 Transforming growth factor- $\beta_1, \beta_2, \beta_3$ Fibroblast growth factor (2,4,8) Bone morphogenetic proteins (2,4,6,7,9,13)

In vitro, IL-1 treatment of articular cartilage or chondrocytes has been shown to stimulate aggrecanase activity (Caterson et al. 2000; Little et al. 1999), induce matrix degradation (Billinghurst et al. 2000; Caterson et al. 2000; Dodge and Poole 1989; Little et al. 1999; Saklatvala et al. 1984; Sandy et al. 1991a; Sandy et al. 1991b) and also inhibit proteoglycan synthesis (Chowdhury et al. 2001; Hauselmann et al. 1996a; Taskiran et al. 1994). In OA joints this cytokine has been found in synovial fluid (Schlaak et al. 1996; Westacott et al. 1990; Wood et al. 1983), synovium (Farahat et al. 1993; Pelletier and Martel-Pelletier 1989) and cartilage (Pelletier and Martel-Pelletier 1989; Tetlow et al. 2001). Receptors for IL-1 have been found on OA chondrocytes at double the normal density in association with an increased sensitivity of the cells to the cytokine in terms of stimulating metalloproteinase secretion (Martel-Pelletier et al. 1992). Further evidence for a role of IL-1 in OA cartilage matrix catabolism comes from studies using animal models of OA. Intra-articular injections of IL-1 have been found to induce proteoglycan loss (Henderson and Pettipher 1989; O'Byrne et al. 1990; Page Thomas et al. 1991) and in two instability models of OA, intra-articular administration of IL-1 receptor antagonist therapy (a competitive inhibitor of

IL-1) has been shown to partially protect against the development of cartilage lesions (Caron et al. 1996; Pelletier et al. 1997).

TNF- α has similar in vitro effects to IL-1 on chondrocytes and cartilage, including stimulation of proteinase activity (Bunning and Russell 1989), matrix degradation (Campbell et al. 1990; Saklatvala 1986) and inhibition of collagen and proteoglycan synthesis (Lefebvre et al. 1990) (Saklatvala 1986). In addition, TNF- α has been localised in OA joints to the synovial fluid (Schlaak et al. 1996; Westacott et al. 1990), synovium (Farahat et al. 1993) and cartilage (Tetlow et al. 2001). The potency of TNF- α appears to be 100-fold to 1000-fold less than that for IL-1 (Lefebvre et al. 1990; Meyer et al. 1990; Saklatvala 1986) but the two cytokines demonstrate synergy to exert cellular effects (Campbell et al. 1990; Meyer et al. 1990; Saklatvala 1986) and, thus, combinations of IL-1 and TNF- α may be important for cartilage destruction (Goldring 2000; Goldring and Goldring 2004).

Some cytokines have anabolic effects. Transforming growth factor- β (TGF- β) is recognised to stimulate chondrocyte proliferation and proteoglycan synthesis (Blumenfeld and Livne 1999; Guerne et al. 1995) and in addition to inhibit IL-1-stimulated proteinase activity (Blumenfeld and Livne 1999), possibly by down-regulation of matrix-degrading proteinases and up-regulation of proteinase inhibitors such as TIMP-1 (Doherty et al. 2004). Insulin-like growth factor-1 (IGF-1) is also anabolic for chondrocyte proteoglycan synthesis (van Osch et al. 1998). It is expressed in fibrillated OA cartilage (Blumenfeld and Livne 1999) and so may form part of the repair response.

Cytokines active in cartilage (Table 1.5) are thought to operate within networks that also involve other mediators such as nitric oxide and prostaglandin E₂ (Goldring and Goldring 2004) in which the overall balance between the activities of catabolic, anti-catabolic, modulatory and anabolic cytokines is important for determining the severity of cartilage damage in OA (Goldring 2000).

Biochemical factors: matrix degradation products

Constituents of matrix that have been degraded also appear to play a part in regulating on-going matrix degradation. Fibronectin is a large glycoprotein that is involved in organising the matrix and helping cell-matrix attachment (Alberts et al. 2002). In vitro, treatment of cartilage explants or chondrocytes with fragments of fibronectin has been found to stimulate MMP production, proteoglycan degradation (Homandberg and Hui 1996; Stanton et al. 2002) and type II collagen degradation (Yasuda and Poole 2002). Depletion of cartilage proteoglycan induced by fibronectin fragments was found to be associated with enhanced release of IL-1 and TNF- α (Homandberg and Hui 1996), suggesting that this catabolic mechanism involves interaction with the cytokine network. Moreover, IL-1 was shown to mediate type II collagen cleavage in this pathway (Yasuda and Poole 2002).

Type II collagen degradation products may be involved in positive cellular feedback mechanisms. Recently, fragments of type II collagen have been found to increase mRNA levels of several MMPs in chondrocytes or cartilage explants (Fichter et al. 2006) and short synthetic peptides of type II collagen have been shown to induce the cleavage of type II collagen and aggrecan in articular cartilage (Yasuda et al. 2006).

Mechanical factors

A further regulatory mechanism affecting chondrocyte metabolism involves mechanical cues (DiMicco et al. 2003). In vitro, proteoglycan synthesis in cartilage explants is inhibited by static compression but stimulated by dynamic compression (Sah et al. 1989). This dependence on dynamic stress and strain for cartilage health is also demonstrated in vivo. In dogs, immobilisation of their joints has been found to cause both reduced synthesis of cartilage proteoglycan and its loss from tissue (Behrens et al. 1989).

Mechanical overload can result in direct effects such as collagen network disruption and matrix fissuring, but physical stimuli are also be important for

modulating cartilage catabolism. Injurious compression of cartilage has been shown to cause cell death, increase proteoglycan release (Quinn et al. 1998) and up-regulate MMP-3 expression (Patwari et al. 2001) and cutting cartilage has been found to induce IL-1 mRNA expression (Gruber et al. 2004). In addition, interaction with the cytokine network may be important for cartilage loss, as suggested by the finding that mechanical injury and IL-1 or TNF- α can act in a synergistic fashion to stimulate proteoglycan degradation (Patwari et al. 2003).

Transduction of mechanical signals to modulate chondrocyte metabolism is thought to be highly dependent on the pericellular matrix (Guilak et al. 2006). Basic fibroblast growth factor (bFGF) is one proposed mechanotransducer in articular cartilage. It has been immuno-localised in the pericellular matrix of articular chondrocytes (Vincent and Saklatvala 2006) and cutting to injure cartilage has been found to release extracellular stores of bFGF that rapidly activated extracellular-signal-regulated kinase (Vincent et al. 2002). Integrins, which are transmembrane cell adhesion proteins that tie the matrix to the cytoskeleton of the cell (Alberts et al. 2002), have also been found to mediate mechanical signals to modify the metabolism of chondrocytes (Chowdhury et al. 2006). Other candidate mechanotransducers include matrilins (Kanbe et al. 2007) and ion channels (Mouw et al. 2007).

1.2.4 Bone changes in OA

Structural and metabolic changes in subchondral bone are clearly evident during the OA process (Burr 2003; Westacott 2003) and the BMD status of patients is associated with the development or progression of disease (Hart et al. 2002; Zhang et al. 2000). Though alterations to subchondral bone often relate in a temporal and topographical fashion to articular cartilage loss, there has been considerable debate about the exact role of subchondral bone in OA pathogenesis (Brandt et al. 2006; Burr 2003). The following sections outline subchondral and systemic bone changes seen in OA, as observed by radiological, histological and biochemical techniques, and discuss theories on

mechanical and biochemical mechanisms by which these changes may relate to articular cartilage loss and the OA process more generally.

Structure and function of subchondral bone

Subchondral bone refers to the layer of lamellar cortical bone underneath articular cartilage and the trabecular cancellous bone buttressing this layer. The cortical bone layer and the calcified cartilage above it form the subchondral plate that functions (i) to physically protect and support the overlying articular cartilage, transmitting forces to the diaphyseal cortex and deforming to increase the contact area under load; and (ii) possibly to provide a source of nutrients to the deeper layers of hyaline cartilage. Whereas subchondral bone is highly vascularised, blood vessels do not normally penetrate through to cartilage (Burr 2003).

Protection of articular cartilage from mechanical damage

Subchondral bone protects the adjacent articular cartilage from damage caused by excessive loads in various ways. Together with other periarticular tissues (capsule, ligament and muscle), subchondral bone has significant force-attenuating properties (Radin and Paul 1970; Radin and Rose 1986). Thus, during joint loading these tissues collectively help to manage potentially injurious peak forces. Cartilage does have damping properties but its overall contribution as a shock absorber is limited by the thinness of the layer (Radin and Rose 1986) and synovial fluid plays no significant role (Radin et al. 1970).

Load transfer from the articular surface to the diaphyseal cortex can generate damaging shear forces at the bone-cartilage interface. The undulating nature of the tide mark at the osteochondral junction transforms these shear forces into and tensile compressive stresses that are more easily managed and less destructive. Furthermore, the presence of subchondral bone also constrains the radial deformation of attached cartilage which imparts some protection against load-induced fissuring to cartilage (Burr 2003).

1.2.4.1 OA subchondral bone: structural changes and possible mechanical implications

Subchondral bone sclerosis, thickness and stiffness

Radiographic subchondral bone sclerosis is a characteristic finding in OA. Thickening of the subchondral cortical plate and subjacent horizontal trabeculae are early changes seen on the radiograph prior to the articular cartilage loss that can be detected by joint space narrowing (Buckland-Wright 2004). Histology also identifies thickened or hypertrophic subchondral bone during the OA process in humans and animal models (Dedrick et al. 1993; Grynpas et al. 1991; Kamibayashi et al. 1995; Li and Aspden 1997; Oettmeier et al. 1992)..

This thickening of subchondral bone is associated with cartilage damage. A study of naturally-occurring OA in cynomolgus monkeys found that cartilage fibrillation in knee joints was unusual without thickening of the subchondral plate and, in addition, the morphological bone changes preceded those found in cartilage (Carlson et al. 1994). A subsequent study using the same primate model of OA demonstrated further evidence for a relationship between subchondral bone and cartilage through the finding that the subchondral plate thickness of the medial tibial plateau increased with worsening severity of articular cartilage lesions (Carlson et al. 1996).

However, in relation to the temporal association during the OA process, other studies have found cartilage changes occurred before bone alterations. Anterior cruciate ligament transection of canine knee joints led to increases in subchondral plate volume within 18 months but these were preceded by mild histological OA changes to articular cartilage that eventually progressed to full thickness loss (Burr 2003). In addition, another mechanical model of OA found that impacting of the patello-femoral joint through a padded interface induced softening of cartilage without underlying subchondral bone thickening (Ewers et al. 2000). Overall, these studies indicate that subchondral bone thickening does

develop during the OA process, but there is conflicting evidence as to whether bone or cartilage structural changes come first.

Mechanical mechanisms may be the link between subchondral bone and cartilage in OA. Over twenty years ago it was hypothesised that OA subchondral bone had areas of increased stiffness that generated destructive forces in the attached articular cartilage, thereby initiating and propagating cartilage damage (Radin and Rose 1986). A further factor compounding this hypothetical destructive process is that load-induced physical forces sustained by individual structures of the joint may be elevated in the diseased joint, as suggested by the finding that OA knee joints exhibit impaired shock-absorbing properties compared to normal knees (Hoshino and Wallace 1987).

Support for the concept of stiffening of subchondral bone in OA has come from studies using animal models of OA. These studies involved repeated impulsive loading of joints and the bone stiffening was found to occur either before or concurrently with cartilage changes (Radin et al. 1978; Simon et al. 1972). On the other hand, more recent studies of late-stage OA in humans have found evidence against the hypothesis. Instead of increased stiffness, thickened subchondral bone from OA femoral heads obtained at arthroplasty has been shown to exhibit reduced stiffness (Li and Aspden 1997) along with decreased mineralisation and material density (Grynpas et al. 1991). Furthermore, in light of the results from an animal study that failed to demonstrate progressive cartilage loss secondary to experimental stiffening of the subchondral plate and the findings of a finite element model that predicted only moderate increases in cartilage stresses secondary to subchondral bone stiffening (Brown et al. 1984), the earlier hypothesis has been revised and, though subchondral bone stiffening is still considered to occur transiently in some situations, it is not thought to be pathophysiologically significant in OA (Burr and Radin 2003).

Microcracks and subchondral cysts

Structural changes to cartilage and subchondral bone can occur following acute trans-articular loading. In vitro structural failure begins with microcracks in the zone of calcified cartilage before gross intra-articular fracture of subchondral bone and overlying cartilage (Vener et al. 1992). Microcracks are also seen in the calcified cartilage of non-diseased human femoral heads (Mori et al. 1993). Their physiological significance in OA is unknown but it is thought that microcracks may initiate vascular invasion of the calcified cartilage, reactivation of the tidemark and enchondral ossification leading to subsequent thinning of the overlying articular cartilage (Burr and Radin 2003). In addition microcracks involving bone are associated with bone remodelling (Bentolila et al. 1998; Burr et al. 1985; Mori and Burr 1993) and may provide physical conduits between cartilage and bone (Burr and Radin 2003).

Subarticular cysts are subchondral lesions that are predominantly located underneath areas of cartilage thinning or loss. They show features of bone necrosis encircled by a rim of reactive new bone and fibrous tissue. These cysts are thought to arise secondary to high intra-articular pressure communicated through defects of the overlying cortical bone, or from abnormal intra-osseous hypertension generated by impaired joint mechanics (Doherty et al. 2004).

Altered joint shape

The bony articular contours can reshape in OA and these alterations are often evident radiographically. At the knee, the femoral and tibial articular surfaces flatten leading to greater congruity of the articular elements, changes that may result from weakened subchondral bone (Buckland-Wright 2004). In advanced stages of the OA process, collapse of the subarticular cancellous bone further deforms the articular surfaces, altering limb alignment (Buckland-Wright 2004). Such changes to the mechanics are likely to lead to the generation of abnormal physical forces in the joint.

Osteophytes are osseous outgrowths at the margin of the articular surface. Though they are considered characteristic manifestations of OA, osteophytes are also seen in the absence of cartilage loss (Moskowitz and Goldberg 1987). Their formation proceeds through an initial chondrophytic stage followed by endochondral calcification and fusion with cortical bone (Hardingham 2004). This process can be stimulated by transforming growth factor- β 1 (van Beuningen et al. 1994) and may also involve basic fibroblast growth factor (Uchino et al. 2000).

It is possible that the osteophyte is an adaptive response of the OA process. Studies during total knee replacement surgery have found that removal of osteophytes increased varus or valgus instability (Pottenger et al. 1990). Thus, marginal osteophytes appear to stabilize OA knees, though their presence can also cause fixed deformities.

Bone bruises

Structural changes to bone are also seen with magnetic resonance imaging (MRI). Early MRI of patients with acute injury and anterior cruciate ligament tear has identified an occult osteochondral lesion (also known as a “bone bruise”) in over 80% of knees (Rosen et al. 1991; Speer et al. 1992). Though not well pathologically characterised, these lesions are thought to represent blood, oedema, hyperaemia and, possibly, microfracture of the trabecular subchondral bone (Mink and Deutsch 1989). Over time, the bone bruise that follows acute anterior cruciate ligament injury can be associated with thinning of adjacent cartilage (Faber et al. 1999). Furthermore at arthroscopy the overlying cartilage is softened and shows histological evidence of damage including degeneration of chondrocytes and loss of proteoglycan (Johnson et al. 1998). Patients with established knee OA also have these “bone marrow oedema” lesions which are associated with painful symptoms (Felson et al. 2001) and an increased risk of progressive disease (Felson et al. 2003).

1.2.4.2 OA subchondral bone: changes in metabolism and molecular composition

Alterations to subchondral bone metabolism provide explanatory mechanisms for some of the observed structural changes described above. In addition, abnormal bone metabolism may directly influence cartilage metabolism through biochemical mechanisms.

(a) Abnormal bone turnover/ remodelling

Bone scan

Convincing evidence for a role of subchondral bone metabolism in OA pathogenesis comes from temporal and topographical associations between metabolic changes seen with bone scintigraphy and the OA process. Bone scintigraphy is an imaging technique that involves the intra-venous injection of a bone-seeking radio-pharmaceutical (usually a technetium-99m-labelled bisphosphonate) followed by its localisation with a gamma camera. The intensity of isotope uptake seen on the scan reflects the rate of bone remodelling (Schauwecker 2003). In patients with generalised nodal OA, bone scintigraphy abnormalities predicted the subsequent detection of radiographic OA change three to five years later, whereas baseline radiographic OA changes on their own had no predictive value (Hutton et al. 1986). Bone scintigraphy has also been found to identify cases of progressive disease in patients with established knee OA. Eighty-eight percent of knees that originally demonstrated severe scan abnormalities subsequently showed decreasing tibio-femoral joint space after five years. In addition, this study found that no knees that scanned normally progressed (Dieppe et al. 1993), indicating a high negative predictive value of the test for progressive disease.

Overall these findings show that physiological changes to bone that are detected by bone scan can precede structural OA changes identified by radiograph in specific joints. Later on in the OA process, the bone scan can

become negative though abnormal structural alterations will persist on the radiograph (Schauwecker 2003).

Biomarkers of bone turnover

Markers of total bone turnover that can be measured in serum, urine or synovial fluid are being evaluated as indicators of subchondral bone formation and resorption (Lohmander and Poole 2003). Several studies have demonstrated increased levels of bone turnover markers in patients following acute joint injury (Lohmander et al. 1996) or in cases of established OA (Astbury et al. 1994; Campion et al. 1989; Seibel et al. 1989; Thompson et al. 1992). However, others have found reduced bone turnover markers in patients with knee OA (Garnero et al. 2001b) and hand OA (Sowers et al. 1999). All of these studies were cross-sectional in design and thus the differences may have arisen due to variation in the rate of bone turnover at different stages of the OA process. A longitudinal study found that bone resorption markers were increased in patients with progressive knee OA but not elevated in those with non-progressive knee OA (Bettica et al. 2002). These findings support the bone scintigraphy data, indicating that bone remodelling appears to be a dynamic process in OA.

There is also evidence at the tissue or cell level that subchondral bone turnover is elevated in OA. Osteocalcin levels (Hilal et al. 1998) and alkaline phosphatase activity (Hilal et al. 1998; Lajeunesse 2004; Mansell et al. 1997; Mansell and Bailey 1998), both markers of bone formation (Westacott 2003), have been found to be elevated. This increased turnover may relate to an altered phenotype of osteoblasts (Hilal et al. 1998) or be driven by elevations in osteoblast-stimulating cytokines, such as insulin-like growth factor and transforming growth factor- β , which have been observed in OA subchondral bone (Hilal et al. 1998; Mansell and Bailey 1998). Transforming growth factor- β may be particularly important for osteophyte formation (van Beuningen et al. 1994).

Histomorphometry

Histomorphometric analyses employing tetracycline labelling indicate that bone remodelling of subchondral plate is one of the earliest changes seen in response to impulsive loading in a rabbit model of OA (Radin et al. 1984). In addition, studies of human samples using static histomorphometry indicate increased bone turnover in the OA subchondral bone from the femoral heads (Grynpas et al. 1991).

Bone remodelling is a well characterised process comprised of coupled events (activation, resorption and then formation) that sequentially occur at the same location. Activation of osteoclasts leads to their resorption of bone, and then during formation, osteoblasts produce an uncalcified matrix, the osteoid. Density and stiffness of bone is endowed by mineralisation of osteoid, a process that initially occurs rapidly but requires six months to a year for full calcification of the matrix (Burr 2004). Thus, during the OA process increased remodelling of subchondral plate will replace old matrix with a new under-mineralised one. Such a scenario provides an explanatory mechanism for the observed decreases in bone density and stiffness in OA subchondral bone (Burr 2004).

(b) Abnormal collagen metabolism in OA subchondral bone

Changes in collagen metabolism are a further indication of subchondral bone abnormality in OA. In humans, the subchondral bone from OA femoral heads shows increases in collagen synthesis and content compared to age-matched controls. Furthermore, this collagen was found to be hypomineralised (Mansell and Bailey 1998). Subsequent studies, using a similar source of tissue, have demonstrated the unusual presence of type I collagen homotrimer (composed of three $\alpha 1$ chains) in addition to the normal type I collagen heterotrimer (two $\alpha 1$ chains and one $\alpha 2$ chain). The significance of this finding is unclear but, drawing on the observations relating to abnormal collagen in osteogenesis imperfecta (a condition characterised by poorly mineralised and highly brittle bone), it has been hypothesised that the presence of type I collagen homotrimer may explain

the hypomineralisation and reduced strength of OA subchondral bone (Bailey et al. 2002).

(c) OA bone cells altering cartilage metabolism

In vitro, bone cells from OA subchondral bone can produce effects on cartilage metabolism. One study isolated cells from the subchondral bone of human OA knee joints that demonstrated medial joint surface cartilage damage and cultured these cells with non-arthritic cartilage (Westacott et al. 1997). Of the bone cell cultures derived from the medial, damaged side of the joint, over a third increased GAG release from cartilage. Over three quarters of the cell cultures established from the lateral side of the same joints altered cartilage metabolism, half of them increasing and half decreasing GAG release. Control bone cell cultures from non-arthritic joints had no effect on GAG release. All cell cultures secreted osteocalcin, indicating osteoblast-like activity and a bone origin. Further work using this co-culture system indicates that aggrecanase activity may be the mechanism for increased GAG release from cartilage caused by the OA bone cells (Diffin et al. 2001).

Recently another in vitro co-culture study found that osteoblasts isolated from sclerotic zones of human OA subchondral bone inhibited aggrecan production and increased MMP-3 and MMP-13 expression from co-cultured human OA chondrocytes (Sanchez et al. 2005). Because osteoblasts from non-sclerotic bone of the same joints did not have these effects on chondrocyte metabolism, the authors concluded that sclerotic OA subchondral osteoblasts could contribute to cartilage degradation by stimulating MMP production and inhibiting aggrecan synthesis.

A biochemical interaction between subchondral bone and cartilage will require a communication between compartments. In normal adult joints this does not appear to exist since vessels do not penetrate the osteochondral junction (Burr 2003) and diffusion through the subchondral plate does not appear to play a role in cartilage nutrition (Ogata et al. 1978). However, during the OA process, a

route for biochemical interaction may be opened up by microcracks in calcified cartilage that have been found in ageing and OA joints (Sokoloff 1993). Additional support for the existence of a biochemical communication between subchondral bone and cartilage in OA comes from the finding that osteocalcin is present in synovial fluid of diseased knee joints (Sharif et al. 1995).

1.2.4.3 Bone mineral density alterations in OA

Measurements of bone mineral density (BMD) that reflect bone mass are used in the diagnosis osteoporosis (Kanis 1994). This is a skeletal disorder characterised by low bone mass and architectural deterioration of bone tissue with a consequent decrease in bone strength and increase in fracture risk (NIH Consensus Development Panel on Osteoporosis Prevention 2001). The possibility of a relationship between OA and osteoporosis has been widely studied. A review of the literature between 1972 and 1996 found that 28 out of 36 publications reported significant increases in bone mass or bone density in OA patients compared to age- and sex-matched controls. Furthermore, correction for body weight, a possible confounding factor, did not change the results in most studies (Dequeker et al. 1996).

More recent longitudinal studies that have further investigated the association between OA and BMD indicate a more complex relationship. Consistent with previous findings, a study of a population-based female cohort found that patients with high BMD or BMD gain at the femoral neck had an increased risk of incident (new onset) knee OA. On the other hand, in patients with established knee OA, low BMD or BMD loss was associated with an increased the risk of radiographic progression (Zhang et al. 2000). Another study confirmed higher BMD measurements (at the hip and spine) in women who went on to develop incident knee OA and also found that low BMD at the hip was weakly related to knee OA progression (Hart et al. 2002).

Further evidence for the role of bone loss in the OA process comes from studies of BMD in the periarticular bone of affected joints (Kannus et al. 1992; Karvonen et al. 1998; Leppala et al. 1999). Ten to eleven years following surgical treatment for anterior cruciate ligament rupture secondary to acute knee injury, BMD measurements have been found to be lower around the injured knee (distal femur, patella and proximal tibia) compared to the uninjured knee (Kannus et al. 1992). BMD was not different between left and right when measured further away from the knee at the femoral neck or calcaneus. This type of bone loss is seen as early as 12 months following knee injury (Leppala et al. 1999) and appears to occur irrespective of whether subjects have osteoporosis based on spine BMD (Karvonen et al. 1998). The findings from these three studies are consistent with the reported lower mineralization and material density of OA subchondral bone that is seen histologically (Gympas et al. 1991; Li and Aspden 1997).

Overall the findings indicate that high BMD is associated with OA, in particular the risk of developing OA. However, once the OA process is established, localised bone loss occurs around affected joints and reductions in BMD are associated with disease progression.

1.2.5 Limitations to interpreting studies of cartilage and bone change in OA

As detailed in sections 1.2.3 and 1.2.4, a great deal of insight has been provided into the underlying mechanisms behind the changes to cartilage and bone in OA. However, many findings have arisen from *in vitro* or *in vivo* studies performed under specific circumstances. Collective interpretation of the evidence base to draw conclusions about common events or pathways of the OA disease process is limited by differences between studies that could have led to different active metabolic processes. Those that are likely to be important include between-study differences in:

- (a) Dominant aetiological factors. Differences in these factors may be difficult to identify, particularly in human studies in which multiple risk factors may be involved.
- (b) Duration of disease activity prior to assessment of outcome measures. OA pathogenesis proceeds slowly and most studies have investigated only a small part of the process. Dominant mechanisms that occur in early OA may be different to those seen in advanced disease.
- (c) Species investigated. Variation in cartilage metabolism is seen between species (Cawston et al. 1998; Hughes et al. 1998).
- (d) Joint sites investigated. Cartilage metabolism varies not only between joints (Eger et al. 2002) but also at different topographical locations within a joint (Barakat et al. 2002; Bayliss et al. 1999).
- (e) Setting for investigation in terms of in vitro vs. in vivo. During in vitro investigation, much of the usual physiological, biochemical and biomechanical environment of the joint tissue of interest is often absent. These environmental factors are important for regulating tissue metabolism in cartilage and the osteochondral unit (discussed in sections 1.2.3.6 and 1.2.4).

Thus, caution should be exercised when extrapolating findings between studies.

1.3 Is there a role for zoledronate as a disease-modifying treatment in osteoarthritis?

Zoledronate and other bisphosphonates are best known clinically as efficacious treatments for various metabolic bone conditions. Interestingly, findings from in vivo and in vitro studies suggest that certain bisphosphonates may be useful as disease-modifying therapies in OA with possible mechanisms of action on cartilage or bone or their interactions in the osteochondral unit.

1.3.1 Zoledronate and other bisphosphonates as therapies for metabolic bone diseases

Over the last four decades, bisphosphonates have become established as effective inhibitors of bone resorption with therapeutic efficacy in the treatment of various metabolic bone disorders such as osteoporosis, Paget's disease, myeloma and bone metastases (Russell 2006).

Chemically, bisphosphonates are stable analogues of inorganic pyrophosphate, a naturally occurring inhibitor of hydroxyapatite crystal formation and dissolution which is thought to be important for regulation of tissue mineralisation (Francis et al. 1969). The phosphonate-carbon-phosphonate structure of bisphosphonates (Fig. 1.3) is important for binding to hydroxyapatite of bone mineral, enabling these compounds to localise to the skeleton. From there they are well placed to exert effects on bone-resorbing osteoclasts (Russell 2006).

The anti-resorptive potency of bisphosphonates has been found to relate to the composition of the R_2 side chain on the bridging carbon atom (Fig. 1.3).

Etidronate and clodronate are early generation bisphosphonates that respectively have a methyl group or chlorine atom at the R_2 position (Fig. 1.4) and were the first to enter clinical use. Newer compounds with side chains containing nitrogen, such as pamidronate and alendronate (Fig. 1.4) were subsequently developed which demonstrated increased anti-resorptive potency. The most potent bisphosphonates were found to be those in which the nitrogen was contained within a heterocyclic ring, for example risedronate and zoledronate (Fig. 1.4) (Russell 2006).

Zoledronate has a R_2 side chain with two nitrogen atoms contained within a heterocyclic ring and in one in vivo model of bone resorption, its potency was observed to be 850 times more than pamidronate and >10000 times more than etidronate (Green et al. 1994). Currently in the UK, zoledronate is licensed for the treatment of Paget's disease of bone, the prevention of skeletal-related



Fig. 1.3. Chemical structures of inorganic pyrophosphate (left) and a generic bisphosphonate (right). From (Russell and Rogers 1999).

Both are shown in their acid forms. The R₂ side chain composition of bisphosphonates is important for determining anti-resorptive potency.

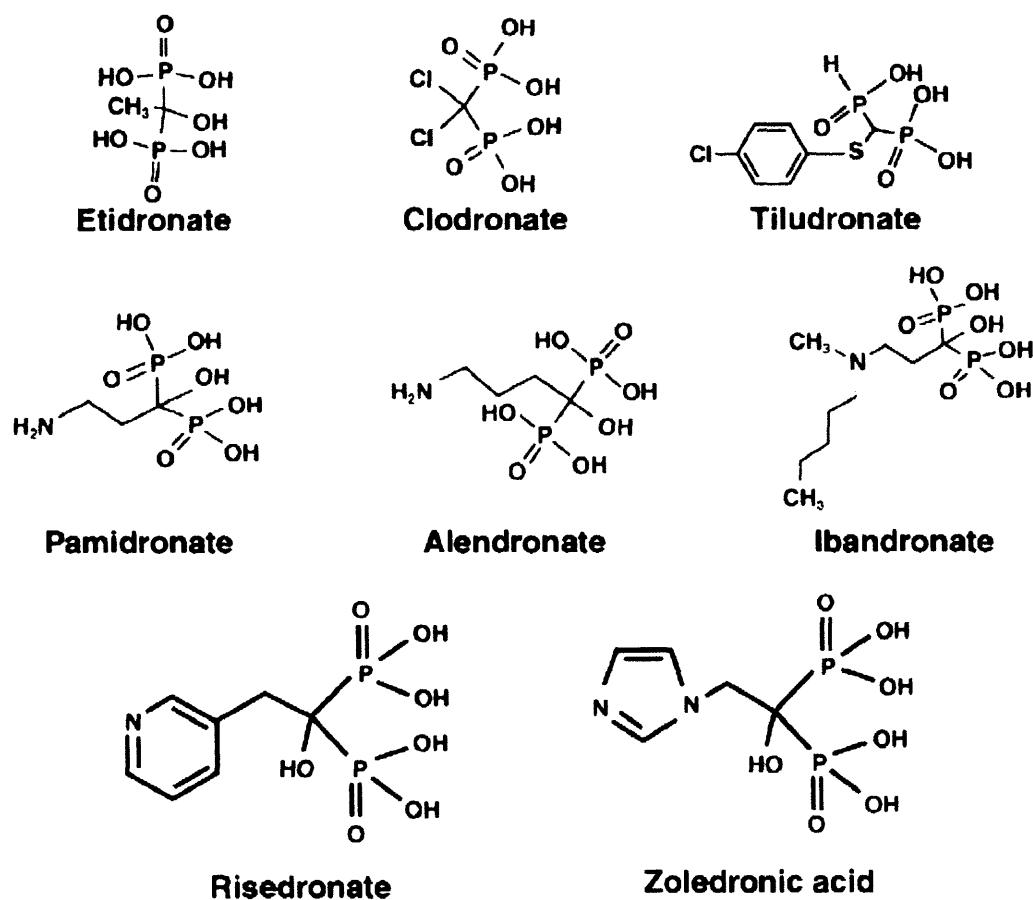


Fig. 1.4. The chemical structures of selected bisphosphonates (shown as acids). From (Green and Rogers 2002).

events in advanced bone malignancies and the treatment of tumour-induced hypercalcaemia (2007a; 2007b). In addition, the results of a recent clinical trial indicate efficacy for zoledronate in the treatment of osteoporosis in post-menopausal women (Black et al. 2007).

Bisphosphonates: mechanisms of bone anti-resorptive activity

Considerable attention has been directed towards elucidating the mechanisms by which bisphosphonates inhibit bone resorption. The principal mechanism is thought to be a direct effect on osteoclasts following cellular uptake by endocytosis (Russell 2006). Cellular effects that have been seen include diminished osteoclast formation, resorptive activity and survival (Benford et al. 2001; Flanagan and Chambers 1991; Hughes et al. 1989; Hughes et al. 1995; Selander et al. 1994; Selander et al. 1996). Results of work to unravel these cellular mechanisms at a biochemical level has led to the proposal that bisphosphonates can be classified into at least two major groups based on modes of intracellular action (Table 1.6) (Russell 2006).

Table 1.6. Classification of bisphosphonates according to mechanism of action. Adapted from (Russell 2006).

Non-nitrogen bisphosphonates		Nitrogen-containing bisphosphonates	
Bisphosphonate	Molecular mechanism of action	Bisphosphonate	Molecular mechanism of action
Etidronate	Incorporated into intracellular analogues of ATP	Pamidronate	Inhibit the prenylation and function of GTP-binding proteins required for osteoclast formation and survival
Clodronate		Alendronate	
Tiludronate		Risedronate	
		Ibandronate	
		Zoledronate	

The first group is comprised of non-nitrogen bisphosphonates such as etidronate and clodronate which have been shown to be metabolised to

analogues of adenosine triphosphate (ATP) (Auriola et al. 1997; Frith et al. 1997; Pelorgeas et al. 1992). These metabolites are non-hydrolysable and accumulate in the cell cytoplasm, a process that is thought to lead to adverse effects on cell metabolism, function and survival (Russell 2006). For example clodronate has been shown to increase osteoclast apoptosis via the intracellular accumulation of a clodronate metabolite (AppCCl₂p) (Frith et al. 2001).

The more potent anti-resorptive bisphosphonates that contain nitrogen form the second group (Table 1.6). These compounds do not appear to be metabolised to analogues of ATP (Frith et al. 1997; Rogers et al. 1994) and act in a different fashion. All nitrogen-containing bisphosphonates have been shown to inhibit the mevalonate pathway (Russell 2006). This pathway is responsible for the biosynthesis of cholesterol and other sterols, and also isoprenoid lipids (such as farnesylidiphosphate and geranylgeranylidiphosphate) that are needed for the post-translational prenylation of small guanine triphosphate (GTP) binding proteins (e.g. Ras, Rab, Rho and Rac). These GTP proteins regulate a variety of cellular functions including membrane ruffling, trafficking of vesicles and apoptosis (Fukuda et al. 2005; Ridley et al. 1992; Zerial and Stenmark 1993). In osteoclasts, Rac1 and Rho have been found to play significant roles in facilitating bone resorptive activity (Fukuda et al. 2005; Zhang et al. 1995).

Thus, the mechanism of anti-resorptive activity seen with the nitrogen-containing bisphosphonates appears to be through interference of protein prenylation and the downstream function of several GTP binding proteins that are important for osteoclast function (Russell 2006). Farnesyl diphosphate synthase, an enzyme within the mevalonate pathway that catalyses the synthesis of isoprenoid lipids, has been identified as the specific target of action. Consistent with this mechanism of action, the abilities of different nitrogen-containing bisphosphonates to inhibit farnesyl diphosphate synthase have been shown to closely correlate to their abilities to inhibit bone resorption *in vivo* (Dunford et al. 2001).

1.3.2 In vivo evidence for zoledronate and other bisphosphonates as disease-modifying treatments in OA

In addition to clinical efficacy in metabolic bone disorders, zoledronate may also have a role in OA. Substantial, though not universal, support for this concept comes from several studies that report on the effects of zoledronate and other bisphosphonates in animal models of OA. Results from clinical trials on the effects of risedronate in knee OA patients have also recently become available (Bingham, III et al. 2006; Spector et al. 2005).

1.3.2.1 Effects of zoledronate on chymopapain-induced cartilage damage in an animal model of OA

Injections of chymopapain into the knee joints of rabbits have been found to cause persistent cartilage and bone changes similar to those seen in OA (Muehleman et al. 2002; Uebelhart et al. 1993; Williams et al. 1993).

Chymopapain is a cysteine endopeptidase of the papain family that has aggrecanolytic activity (Dekeyser et al. 1995) but does not cause direct damage to collagen fibres (Postacchini et al. 1982).

Cartilage and bone changes have been characterised for the OA model. Two days after intra-articular chymopapain injections, pronounced loss of cartilage proteoglycan occurs followed by fibrillation of the articular surface and chondrocyte loss at three weeks (Uebelhart et al. 1993; Williams et al. 1988). Subsequently, at six months, the cartilage becomes eroded in loaded regions and marginal osteophytes develop. Associated with these changes there are increases in urinary levels of lysyl pyridinoline (LP) and hydroxylysyl pyridinoline (HP) crosslinks of collagen (Muehleman et al. 2002; Uebelhart et al. 1993). LP cross-links appear to be fairly specific for collagen in bone whereas HP collagen cross-links are found in bone and cartilage (Eyre et al. 1988). Thus, the elevated urinary levels of LP and HP cross-links are considered to reflect increased collagen catabolism in bone and bone or cartilage respectively (Uebelhart et al. 1993).

In a controlled-study that used this model of cartilage damage, zoledronate treatment has been found to partially reduce levels of grossly- and histologically-detectable cartilage degeneration (Muehleman et al. 2002). Commencing 24 hours before intra-articular chymopapain, zoledronate 10 µg/kg was administered to rabbits by subcutaneous injection three times a week, dosing that approximates to the monthly intravenous zoledronate 4 mg regimen that is used clinically in humans (personal communication from Dr. J.R. Green, Novartis Pharma AG). Animals were sacrificed for histological and biochemical analyses 28 days and 56 days post chymopapain and the results for these two time points were reported together. Though all chymopapain-injected knees, whether the animals were zoledronate-treated or not, demonstrated cartilage damage compared to uninjected control knees, partial reductions for some outcome measures were observed with zoledronate treatment.

A gross visual grade was used to measure cartilage fibrillation, fissuring and erosion, and also osteophytes in the knee joints (Muehleman et al. 2002). Chymopapain injections led to significant increases in joint damage assessed by the visual grade and this effect was partly attenuated by zoledronate treatment. Articular cartilage was also scored for microscopic histological change. On a 15-point scale that assessed surface integrity, cell clone formation and cellularity (15 points representing the highest level of degenerative change), the mean score for tibial cartilage was lower for chymopapain-injected/ zoledronate treated rabbits than chymopapain-injected rabbits (mean scores 5.8 and 8.6 respectively; P=0.001 for the difference). In addition, for patella cartilage there was a trend for a lower score in the chymopapain-injected/ zoledronate treated group compared to the chymopapain-injected group (mean scores 6.5 and 7.3 respectively; P=0.07 for the difference).

Biochemical analyses revealed a trend for improved cartilage proteoglycan retention with zoledronate treatment (Muehleman et al. 2002). Chymopapain-

injected/ zoledronate-treated animals demonstrated a 21% reduction in cartilage proteoglycan content of injected knees compared to 28% for chymopapain-injected animals ($P=0.12$ for difference between groups). However, rises in serum levels of keratan sulphate (a proposed measure of cartilage proteoglycan degradation (Williams et al. 1988)) with chymopapain injections were not altered by zoledronate treatment.

In association with these histological and biochemical findings, the study also found indirect evidence for inhibition of bone remodelling with zoledronate treatment (Muehleman et al. 2002). Urinary levels of LP cross-links rose with chymopapain injections but not with concomitant zoledronate treatment.

Based on the study findings, the authors hypothesised that inhibition of bone remodelling by zoledronate in the model might be the mechanism for the observed protective effects on cartilage damage (Muehleman et al. 2002). However, the pathophysiological relationship between subchondral bone remodelling and cartilage degradation in the model is not clear (discussed in section 1.2.4). Clearly another possibility is inhibition of chymopapain proteolytic activity, though such an ability has not been reported in the MEROPS peptidase database (Rawlings et al. 2006). Alternatively, the mechanism could be a direct effect by zoledronate on cartilage metabolism (further discussed below).

1.3.2.2 Effects of zoledronate in a cruciate-deficient animal model of OA

Surgical transection of the anterior cruciate ligament of the stifle joint in dogs causes joint instability and provides an animal model in which persistent OA-like lesions develop (Brandt et al. 1991). A few weeks after ligament transection, the articular cartilage begins to swell. Cartilage degeneration subsequently develops with fibrillation, loss of surface tissue and chondrocyte cloning. Marked osteophyte formation is apparent at about three months (Griffiths and Schrier 2003) and eventually cartilage ulceration progresses to full thickness loss (Brandt et al. 1991). Other changes that have been observed in unstable

knees in the model include increases in bone turnover (Brandt et al. 1997), decreases in periarticular BMD indicative of bone loss (Agnello et al. 2005; Behets et al. 2004; Boyd et al. 2000) and mechanical weakening of periarticular cancellous bone (Wohl et al. 2001).

The effects of zoledronate treatment have been evaluated in this model of OA (Agnello et al. 2005). Though articular cartilage changes were not assessed, subcutaneous zoledronate treatment was found to inhibit elevations in serum osteocalcin and bone-specific alkaline phosphatase (markers of systemic bone remodelling) and to prevent decreases in periarticular BMD that occurred following anterior cruciate transection.

1.3.2.3 Other bisphosphonates and in vivo models of OA

Substantial attention has focused on risedronate as a possible disease-modifying treatment in OA. The bisphosphonate was initially evaluated in an animal OA model (Meyer et al. 2001a; Meyer et al. 2001b). The Duncan-Hartley strain guinea pigs that were used in the studies are known to develop spontaneous cartilage degeneration (Bendele and Hulman 1988) preceded by subchondral bone remodelling (Griffiths and Schrier 2003; Quasnicka et al. 2006). Treatment with risedronate or with bisphosphonates structurally similar to risedronate was found to slow progression of macroscopically-evident cartilage degeneration and osteophyte formation in Duncan-Hartley guinea pigs (Meyer et al. 2001a; Meyer et al. 2001b). Interestingly, alendronate and other bisphosphonates structurally dissimilar to risedronate did not demonstrate treatment effects in this model. Risedronate has also been found to protect against periarticular BMD loss and to partly attenuate the deterioration in some of the mechanical properties of bone and ligament in the knee joints of rabbits with instability induced by ACL transection (Doschak et al. 2004). These favourable results for risedronate have been followed by studies in human OA.

A British one-year prospective randomised placebo-controlled trial investigated the effects of risedronate in 284 patients with OA of the medial compartment of the knee (Spector et al. 2005). Patients taking the higher dose of risedronate tested (15 mg daily) showed improvement in symptoms and function as assessed by the Western Ontario and McMaster Universities (WOMAC) OA index, the patient global assessment and the use of walking aids. In addition a trend towards attenuation of joint-space narrowing was observed. Though numbers were small, 8% of patients receiving placebo (n=7) and 4% of those receiving 5 mg risedronate daily (n=4) demonstrated radiographic disease progression compared with 1% (n=1) of patients receiving 15 mg risedronate ($P=0.067$). Radiographic disease progression was defined as joint-space narrowing of ≥ 0.75 mm or loss of joint space of $\geq 25\%$. Associated with these observations, risedronate treatment reduced urinary levels of N-terminal cross-linking telopeptide of type I collagen (NTX-I) and urinary levels of C-terminal cross-linking telopeptide of type II collagen (CTX-II). NTX-I and CTX-II are proposed markers of bone resorption (Pagani et al. 2005) and cartilage degradation respectively (Christgau et al. 2001; Lohmander and Poole 2003).

However, these encouraging results were not reproduced in a large 2-year trial that used a similar protocol involving nearly 2500 patients with medial compartment knee OA from North America and the European Union (KOSTAR study)(Bingham, III et al. 2006). Risedronate treatment did cause a dose-dependent reduction in urinary CTX-II levels but, for the primary outcomes investigated, risedronate (5 mg daily, 15 mg daily, 35 mg weekly or 50 mg weekly) compared to placebo did not improve symptoms and signs of OA or reduce radiographic progression of knee OA.

A more detailed analysis of the patients from the North American arm of the KOSTAR study on the effects of risedronate on subchondral bone has been reported in a separate study (Buckland-Wright et al. 2007). In patients with progressive disease (defined in the study as joint-space narrowing ≥ 0.6 mm over 2 years in the OA knee), higher doses of risedronate treatment were found

to inhibit radiographically-measured trabecular bone loss in the subchondral region of the diseased medial compartment of the tibia.

Other bisphosphonates have been evaluated using animal models of OA with conflicting findings for effects on cartilage degradation. Etidronate given to SRT/ORT mice, an inbred strain which commonly develops a severe form of OA (Griffiths and Schrier 2003), did not influence the incidence or severity of histologically-assessed cartilage degeneration (Walton 1981). The bisphosphonate NE-10035 (2-[acetylthio]ethylidene-1, 1-bisphosphonate) has been tested in the canine cruciate deficient model of OA (Myers et al. 1999). Though NE-10035 effectively reduced subchondral bone turnover, it had no effect on osteophyte formation or the severity of OA cartilage changes. However the authors did note that the cartilage degeneration observed in the study was mild and the possibility of an inhibitory effect on cartilage damage in a model displaying more severe cartilage degradation remained.

Disease-modifying effects have been demonstrated with alendronate treatment in the rat ACL transection model of OA (Hayami et al. 2004). This bisphosphonate was found to prevent osteophyte formation, inhibit bone turnover (assessed by bone histomorphometry) and partially protect against the development of histologically-observed cartilage degeneration. Consistent with these structure-modifying effects, alendronate also reduced urinary levels of CTX-II and C-terminal telopeptide of type I collagen (CTX-I; a marker of bone resorption (Pagani et al. 2005)).

1.3.2.4 Zoledronate and other bisphosphonates in inflammatory arthritis

Following encouraging findings from animal models of inflammatory arthritis, there has been recent interest in the use of bisphosphonates to reduce the development of bone erosions in inflammatory arthritic conditions such as rheumatoid arthritis (Breuil and Euller-Ziegler 2006; Goldring and Gravallese 2004). In addition, zoledronate has been found to partly inhibit cartilage

degradation along with decreasing focal bone damage in transgenic mice with tumour necrosis factor-mediated arthritis (Herrak et al. 2004) and in rabbits with carrageenan-induced inflammatory arthritis (Podworny et al. 1999).

However, several clinical trials with bisphosphonates in patients with rheumatoid arthritis have found more equivocal results. No evidence for efficacy with respect to preventing the progression of focal bone destruction was observed for pamidronate in two studies (Lodder et al. 2003; Ralston et al. 1989) and etidronate in one study (Valleala et al. 2003) though another trial demonstrated a beneficial effect for pamidronate (Maccagno et al. 1994). In addition, a small study of 39 rheumatoid arthritis patients has found that zoledronate treatment can prevent the development of new bone erosions (Jarrett et al. 2006).

Extrapolation of the findings arising from studies of inflammatory arthritis to OA is questionable because the underlying pathogenic mechanisms are different. By definition there is often intense synovial inflammation driving the pathology in inflammatory arthritis. This is not usually present in OA. However, the beneficial effect of zoledronate on cartilage damage in the animal models of inflammatory arthritis was unlikely to have been due to an anti-inflammatory action since no accompanying reductions in synovitis were observed (Herrak et al. 2004; Podworny et al. 1999), suggesting an alternative mechanism such as a direct effect on cartilage metabolism. In both inflammatory arthritis and OA, MMPs and aggrecanases are key mediators of cartilage matrix destruction (Clark and Parker 2003; Elliott and Cawston 2001). Thus, the findings from these studies of zoledronate in models of inflammatory arthritis (Herrak et al. 2004; Podworny et al. 1999) may be relevant for considering a role for the bisphosphonate in OA.

1.3.3 Exploring potential mechanisms of action of zoledronate as a disease-modifying treatment in OA

Understanding the mechanisms by which zoledronate might be able to modify the OA disease process will support a role for the bisphosphonate as a treatment for OA. In the subsequent discussion the following possible targets for zoledronate in the OA joint will be considered:

- (a) Inhibition of bone remodelling
- (b) A direct effect on cartilage metabolism

Although findings with respect to bisphosphonates other than zoledronate will continue to be discussed, it is useful to bear in mind that disease-modifying effects seen with bisphosphonate treatment in one animal OA model did not relate to bisphosphonate anti-resorptive potency (Meyer et al. 2001a; Meyer et al. 2001b). The absence of such an association indicates that a bone effect is a less likely mechanism, making an effect on cartilage the more attractive underlying mechanism of action.

1.3.3.1 Inhibition of bone turnover by zoledronate: a possible mechanism for disease-modification in OA?

Structural and metabolic changes to subchondral bone are clearly evident in the OA joint though their pathophysiological relationship to cartilage loss and the OA process are not well understood (discussed in section 1.2.4). Furthermore BMD appears to relate to OA incidence and progression. Zoledronate and other bisphosphonates have well-known effects on bone, and these may beneficially modify the OA process. In this section, the effects of bisphosphonate on bone and their possible implications for the OA joint are discussed.

Is inhibiting bone remodelling in OA a useful effect?

OA joints that demonstrate increased bone turnover on bone scintigraphy have a higher risk of disease progression (Dieppe et al. 1993; Hutton et al. 1986).

Thus, using a bisphosphonate such as zoledronate to inhibit bone remodelling and modify this risk factor may retard OA disease progression.

Support for this concept comes from the numerous *in vivo* studies that evaluated zoledronate and other bisphosphonates in models of OA which found favourable treatment effects on OA cartilage and subchondral bone (Agnello et al. 2005; Buckland-Wright et al. 2007; Doschak et al. 2004; Hayami et al. 2004; Meyer et al. 2001a; Meyer et al. 2001b; Muehleman et al. 2002; Spector et al. 2005). In addition, calcitonin, another bone anti-resorptive agent, has been found to reduce the severity of OA cartilage lesions in the canine ACL transection OA model (Manicourt et al. 1999) and to attenuate cartilage and subchondral bone changes in the rabbit ACL transection OA model (Papaioannou et al. 2007)

Ultimately, whether inhibition of bone remodelling is beneficial in OA will depend on the role of bone remodelling during OA pathogenesis. This remains an area that is not well understood. However, it is interesting to consider how modifying bone metabolism might affect the OA process.

Implications of effects on BMD and subchondral bone structure

Zoledronate treatment leads to sustained increases in BMD in post-menopausal women with osteoporosis, along with reducing the risk of fracture (Black et al. 2007). It is likely that the bisphosphonate would also increase BMD in OA patients. If the BMD status of patients that has been found to be associated with OA according to epidemiological data (discussed in section 1.2.4.3) turns out to be a causal risk factor for OA, then zoledronate treatment to increase BMD could increase the risk of incident OA but, on the other hand, reduce the risk of disease progression in established OA.

With regard to local bone loss, subcutaneously-administered zoledronate was found to prevent significant BMD decreases in periarticular bone in the canine ACL transection model of OA, (Agnello et al. 2005). Additionally, zoledronate

has been observed to increase the mechanical strength of long bones of ovariectomised rats (Hornby et al. 2003) and if such effects also occurred in periarticular bone, the subchondral plate might be protected from acute structural failure and the development of microcracks. However, the pathophysiological significance of such structural changes in OA is not clear. Strengthening OA subchondral bone may also prevent articular surface deformity and limb malalignment, changes that are thought to arise from collapse of weakened subarticular cancellous bone (Buckland-Wright 2004).

Bisphosphonates may also reduce osteophyte formation. In the rat ACL transection model of OA, alendronate was found to dose-dependently decrease the incidence and area of osteophytes (Hayami et al. 2004). In addition, risedronate reduced the size of osteophytes that developed in the Duncan-Hartley guinea pig OA model (Meyer et al. 2001b). However, treatment with the bisphosphonate NE-10035 in the canine ACL transection OA model (Myers et al. 1999) or risedronate in patients with knee OA (Bingham, III et al. 2006) did not produce effects on osteophyte formation. Inhibition of osteophyte formation will clearly modify one of the characteristic features of OA, but because the osteophyte might actually improve joint instability (Pottenger et al. 1990), this disease-modifying effect may not be beneficial for the OA process.

Potential effects of zoledronate on a possible biochemical interaction between OA subchondral bone and articular cartilage

Osteoblasts from OA subchondral bone can biochemically stimulate GAG release or inhibit aggrecan synthesis in cartilage, and so may be important for cartilage degradation (Sanchez et al. 2005; Westacott et al. 1997). If this abnormal osteoblastic activity occurs as part of bone remodelling, then a potent anti-resorptive therapy such as zoledronate could have beneficial effects. Another possibility is a direct effect on the OA osteoblasts since zoledronate has been found to directly act on osteoblasts to inhibit their proliferation (Fromigue and Body 2002; Reinholtz et al. 2000).

1.3.3.2 Does zoledronate have effects on cartilage metabolism?

Can zoledronate localise to cartilage?

Localisation of zoledronate to cartilage has not been reported, but one theoretical route for systemically-administered zoledronate to reach cartilage is via bone. The bisphosphonate has a high adsorption affinity for hydroxyapatite (Nancollas et al. 2006) and is avidly taken up by bone following intravenous administration (Green and Rogers 2002). Because bisphosphonates in general bind preferentially to areas of bone which have high turnover rates (Lin 1996), such as OA subchondral bone (section 1.2.4.2), it is likely that zoledronate can localise to the bone underlying articular cartilage in the OA joint. Possible routes from subchondral bone to cartilage include the synovial fluid and microcracks in OA calcified cartilage (section 1.2.4.2).

Alternatively, the blood circulation could deliver the bisphosphonate directly to cartilage in diseased joints. Though blood vessels do not penetrate from subchondral bone through to cartilage in normal synovial joints, capillary invasion of the tidemark is seen in OA (Sokoloff 1993). Overall, the existence of these possible access routes is consistent with the concept that the protective effects on cartilage damage with zoledronate treatment observed in an animal OA model (Muehleman et al. 2002) could have occurred through a direct action on cartilage.

Effects of bisphosphonates on chondrocyte synthetic function and survival

In vitro work has demonstrated direct effects of bisphosphonates on cartilage or chondrocyte matrix synthesis, though there is some discordance between studies. Etidronate and clodronate (at concentrations ranging from 10^{-6} M to 5×10^{-4} M) were shown to produce reversible inhibition of GAG synthesis in canine articular cartilage explants (Palmoski and Brandt 1978). However, for rabbit articular chondrocytes, clodronate (2.5×10^{-5} M and 2.5×10^{-4} M), but not

etidronate (2.5×10^{-4} M), was found to increase GAG and collagen synthesis (Guenther et al. 1979; Guenther et al. 1981). In addition, mild inhibition of cell proliferation was observed for 2.5×10^{-4} M clodronate and 2.5×10^{-4} M etidronate (Guenther et al. 1979).

Chondrocyte survival appears to be promoted by some bisphosphonates under certain conditions. In an in vitro study of bovine articular chondrocytes, incubation with glucocorticoid dexamethasone was used to reduce cell viability, to increase cell apoptosis and to inhibit cell proliferation in the cultures (Van Offel et al. 2002). Each of these detrimental effects on chondrocytes was partially prevented by co-treatment with pamidronate (10^{-6} M) or risedronate (10^{-8} M or 10^{-6} M) but not with clodronate (Van Offel et al. 2002). Higher concentrations of the bisphosphonates were not protective and, moreover, at 10^{-4} M and above, the bisphosphonates were found to reduce cell viability or proliferation.

Effects of zoledronate and other bisphosphonates on chondrocyte-mediated degradation of cartilage matrix

Evidence from several studies points towards cartilage matrix catabolism as a potential target for zoledronate. Reductions in urinary CTX-II levels have been observed in patients with Paget's disease of bone following intravenous zoledronate (Garnero et al. 2001a). Though the OA status of the subjects was unknown, and so it is not clear whether the effects might relate to normal or OA cartilage, the findings do suggest that the bisphosphonate has an ability to inhibit type II collagen degradation. Furthermore, the observed reduction in urinary CTX-II was rapid (within five days) and transient which points towards a direct effect of zoledronate on cartilage rather than on subchondral bone with consequent changes to cartilage metabolism. This is because (i) five days is too early to see structural alterations to subchondral bone that could have had secondary effects on cartilage; and (ii) an effect that arose from inhibition of bone remodelling should have been persistent.

In agreement with a possible effect of bisphosphonates in general on collagen II metabolism, decreases urinary CTX-II have also been observed following risedronate treatment in knee OA patients (Bingham, III et al. 2006; Spector et al. 2005). One caveat about these studies involving urinary CTX-II assays is that there may be unknown factors affecting systemic metabolism of the marker prior to its measurement in urine which could complicate interpretation (Lohmander and Poole 2003).

Direct evidence for an effect on cartilage matrix catabolism comes from in vitro work. Three reported studies employed a similar culture model in which an IL-1-like factor (either derived from mononuclear cells, macrophages, or synovial tissue) was used to stimulate proteinase production by articular chondrocytes (Emonds-Alt et al. 1985; Evequoz et al. 1985; McGuire et al. 1982). Cultures were also treated with the bisphosphonates of interest and at the end of the culture period proteinase activity was assayed in the chondrocyte-conditioned culture medium. Findings indicate that chondrocyte-derived proteinase activity can be influenced by bisphosphonates, though the reports are somewhat conflicting as to the direction of effect.

Addition of etidronate 2.5×10^{-5} M or pamidronate 2.5×10^{-6} M was found to enhance the collagenase activity produced by stimulated rabbit chondrocytes, though treatment effects were not seen with clodronate (2.5×10^{-5} M or 2.5×10^{-4} M) or etidronate at a higher concentration (2.5×10^{-4} M) (Evequoz et al. 1985). Another study also evaluated etidronate and clodronate but used human chondrocytes (McGuire et al. 1982). However, in these experiments, 2.5×10^{-4} M etidronate or 2.5×10^{-4} M clodronate were both found to inhibit stimulated collagenase activity. A limitation of both studies is that specific data on possible cytotoxic effects of the bisphosphonates were not shown and, thus, cell death can not be excluded as the mechanism for any observed inhibitory effects.

From a third study with similar methodology, tiludronate (10^{-5} M to 5×10^{-4} M) was seen to dose-dependently inhibit proteinase activity (against collagen,

proteoglycan and casein) produced by stimulated rabbit chondrocytes (Emonds-Alt et al. 1985). Etidronate (10^{-4} M to 10^{-3} M) also inhibited these enzyme activities but was much less potent. In this study, the bisphosphonates at the concentrations tested did not induce chondrocyte toxicity.

In addition, the effects of etidronate and pamidronate on proteoglycan degradation in bovine nasal septum cartilage have been examined (Couchman and Sheppeard 1986). Following culture of the bisphosphonates with cartilage, the amount of GAG released from cartilage into the medium was measured. In this culture model, 10^{-5} M pamidronate increased GAG release, though no effect was observed with 10^{-5} M etidronate.

It is not clear why the results are discrepant between in vitro studies. Factors that may have been significant include (i) inter-species differences in chondrocyte metabolism (Hughes et al. 1998), (ii) differences in metabolism between nasal and articular cartilage (Caterson et al. 2000), (iii) differential effects between bisphosphonates and (iv) differing bisphosphonate treatment concentrations between studies in conjunction with possible cytotoxic effects at high bisphosphonate concentrations (Van Offel et al. 2002). Nonetheless, there are findings to suggest that certain bisphosphonates (etidronate, clodronate and tiludronate) can favourably inhibit chondrocyte-mediated catabolism of matrix (Emonds-Alt et al. 1985; McGuire et al. 1982).

Effects of zoledronate and other bisphosphonates on MMP activity

At the biochemical level, inhibition of the matrix-degrading enzymes, the MMPs and the aggrecanases, is an attractive therapeutic strategy to prevent cartilage loss in arthritic conditions including OA (Clark and Parker 2003). Several reports by a Finnish research group indicate that zoledronate and other bisphosphonates can directly reduce MMP activity in vitro (Heikkila et al. 2002; Konttinen et al. 1999; Teronen et al. 1997a; Teronen et al. 1997b; Teronen et al. 1999). These studies employed cell-free enzyme activity assays using

various substrates such as casein, pro-urokinase, gelatine and type I collagen, though not aggrecan or type II collagen.

Zoledronate (concentration range 2×10^{-5} M to 10^{-3} M) was found to inhibit human MMPs-3, 8, 13 and 20 in a concentration-dependent fashion with fifty percent inhibition (IC_{50}) of MMP-20 activity occurring at zoledronate concentrations between 5×10^{-5} M to 10^{-4} M (Heikkila et al. 2002). In the same study, similar inhibitory effects were seen for clodronate and alendronate (against MMPs-1, 2, 3, 8, 9 and 13), and pamidronate (against MMPs-3, 8, 13 and 20). In addition, increasing the calcium ion concentration in the assay buffer attenuated some of the inhibitory abilities of clodronate and alendronate suggesting that the mechanism of MMP inhibition involved interaction between the bisphosphonate and divalent cations, such as the zinc ion bound at the MMP catalytic site.

Separate studies reported by the same group also found inhibitory effects of several bisphosphonates on MMP activity. Alendronate reduced MMP-13 activity (IC_{50} between 5×10^{-4} M and 7.5×10^{-4} M) (Konttinen et al. 1999), and clodronate inhibited the activities of MMP-1 (IC_{50} 1.5×10^{-4} M) (Teronen et al. 1997b) and MMP-8 (IC_{50} 1.5×10^{-4} M) (Teronen et al. 1997a). In addition, zoledronate reduced the activities of MMPs-3, 12, 13 and 20 in a concentration-dependent fashion with IC_{50} s that ranged from 5×10^{-5} M to 1.5×10^{-4} M (Teronen et al. 1999).

Though currently there are no reports on the effects of bisphosphonates on aggrecanase activity, metal-chelating chemicals are known to inhibit the activities of both MMPs and aggrecanases (Hughes et al. 1998). This is understandable since both families of enzymes contain zinc in their metallo-proteinase catalytic domain (Murphy and Lee 2005). Thus, in theory, bisphosphonates should be able to inhibit aggrecanase activity.

1.3.4 Summary of evidence. Direction for investigation

In vivo data largely support the concept that there is a role for zoledronate as a disease-modifying treatment in OA. Studies that have evaluated zoledronate in models of OA and inflammatory arthritis are summarised in Table 1.7. In addition, Table 1.8 summarises the in vivo data on other bisphosphonates in models of OA. Despite their promising findings, these in vivo studies are not able to conclude on underlying mechanisms of action. Exploring potential mechanisms of action would provide additional insight towards defining a possible role for zoledronate as a disease-modifying treatment in OA.

Clearly zoledronate has well known actions on bone metabolism but an alternative target is cartilage metabolism. In cartilage, loss of the principal GAG-bearing proteoglycan (aggrecan) from cartilage is a well characterised process

Table 1.7. Effects of zoledronate in animal models of osteoarthritis and inflammatory arthritis.

Model of OA or IA		Efficacious bisphosphonate dose	Effects observed	Reference
Chymopapain-induced cartilage damage in rabbits	OA	Subcutaneous injections; 10 µg/kg 3x per week	<ul style="list-style-type: none"> Partially reduced cartilage damage assessed by histology Trend for improved cartilage proteoglycan retention Prevented rise in urinary lysyl pyridinoline collagen cross-links (marker of bone turnover) 	(Muehleman et al. 2002)
ACL transection in dogs	OA	Subcutaneous injections; 10 µg/kg or 25 µg/kg every 90 days	<ul style="list-style-type: none"> Prevented peri-articular BMD decreases Inhibited rise in serum osteocalcin (marker of osteoblast activity) 	(Agnello et al. 2005)
Collagen-induced arthritis in rats	IA	Subcutaneous injections; ≥10 µg/kg as a single dose	<ul style="list-style-type: none"> Reduced bone erosions Reduced juxta-articular trabecular bone loss Reduced serum type I collagen cross-link levels (marker of bone resorption) 	(Sims et al. 2004)
Human tumour necrosis factor transgenic mice	IA	Intra-peritoneal injections; 100 µg/kg as a single dose or 5x per week	<ul style="list-style-type: none"> Prevented bone erosions Partially reduced cartilage damage assessed by histology (repeated doses only) Reduced serum lysyl pyridinoline collagen cross-link levels and serum osteocalcin levels 	(Herrak et al. 2004)
Carageenan-induced inflammatory arthritis in rabbits	IA	Subcutaneous injections; 10 µg/kg 3x per week	<ul style="list-style-type: none"> Preserved subchondral bone integrity assessed by histology Partially reduced cartilage degradation assessed by histology Trend for improved cartilage proteoglycan retention 	(Podworny et al. 1999)

Table 1.8. Effects of bisphosphonates (other than zoledronate) in OA animal models and OA clinical trials.

Bisphosphonate evaluated	OA model	Bisphosphonate dose	Effects observed	Reference
Risedronate	Duncan-Hartley guinea pigs	Subcutaneous: 12 µg/kg or 30 µg/kg 5x per week	<ul style="list-style-type: none"> Reduced grossly-visible cartilage lesion size Reduced grossly-visible osteophyte size 	(Meyer et al. 2001b)
	ACL transection in rabbits	Subcutaneous: 10 µg/kg daily	<ul style="list-style-type: none"> Prevented periarticular BMD decreases Partially conserved mechanical properties of peri-articular bone and the medial collateral ligament 	(Doschak et al. 2004)
	Human knee OA (clinical trial)	Oral: 5 mg or 15 mg daily	<ul style="list-style-type: none"> Improved symptoms and function (15 mg dose) Trend for reduced radiographic joint space narrowing Reduced urinary CTX-II levels (marker of cartilage degradation) Reduced urinary NTX-I levels (marker of bone resorption) 	(Spector et al. 2005)
	Human knee OA* (clinical trial)	Oral: 5 mg/day, 15 mg/day, 35 mg/week or 50 mg/week	<ul style="list-style-type: none"> No treatment effects demonstrated for symptoms/signs or radiographic joint space narrowing Reduced levels of urinary CTX-II Reduced levels of urinary NTX-I 	(Bingham, III et al. 2006)
	Human knee OA* (clinical trial)	Oral: 5 mg/day, 15 mg/day, or 50 mg/week	<ul style="list-style-type: none"> In patients with marked cartilage loss (radiographic joint space narrowing ≥ 0.6 mm), conserved subchondral bone structure (15 mg/day and 50 mg/week dosing) 	(Buckland-Wright et al. 2007)
	SRT/ORT mice	Subcutaneous: 5 mg/kg or 50 mg/kg	<ul style="list-style-type: none"> No treatment effect on articular cartilage degeneration assessed by histology Reduced levels of bone resorption 	(Walton 1981)
NE-10035	ACL transection in dogs	Subcutaneous: 5-10 mg/kg 5x per week	<ul style="list-style-type: none"> Reduced subchondral bone turnover assessed by histomorphometry No treatment effect on osteophyte formation or OA cartilage changes assessed by histology 	(Myers et al. 1999)
Alendronate	ACL transection in rats	Subcutaneous: 15 µg/kg or 120 µg/kg twice a week	<ul style="list-style-type: none"> Partially reduced cartilage damage assessed by histology Prevented osteophyte formation (120 µg/kg dose) Prevented rise in urinary CTX-I and CTX-II 	(Hayami et al. 2004)

*These two studies involved the same group of patients. OA= osteoarthritis; ACL= anterior cruciate ligament; BMD= bone mineral density; CTX-I= C-terminal cross-linking telopeptide of type I collagen; CTX-II= C-terminal cross-linking telopeptide of type II collagen; NTX-I= N-terminal cross-linking telopeptide of type I collagen

Table 1.9. Direct effects of bisphosphonates on cartilage metabolism

Metabolic process in cartilage	Effect observed with various bisphosphonates			
	Bisphosphonate	Effect	Bisphosphonate concentration with activity	Reference
Chondrocyte survival or proliferation	Clodronate	Reduced	10^{-4} M to 10^{-3} M	(Van Offel et al. 2002)
	Pamidronate	Reduced	10^{-3} M	(Van Offel et al. 2002)
	Risedronate	Reduced	10^{-3} M	(Van Offel et al. 2002)
Matrix synthesis-GAG	Etidronate	Reduced	10^{-6} M to 5×10^{-4} M	(Palmoski and Brandt 1978)
	Clodronate	Reduced	10^{-6} M to 5×10^{-4} M	(Palmoski and Brandt 1978)
	Clodronate	Increased	2.5×10^{-5} M to 2.5×10^{-4} M	(Guenther et al. 1979)
Matrix synthesis-Collagen	Clodronate	Increased	2.5×10^{-5} M to 2.5×10^{-4} M	(Guenther et al. 1981)
Chondrocyte-mediated proteinase activity	Etidronate	Increased	2.5×10^{-5} M	(Evequoz et al. 1985)
		Reduced	2.5×10^{-4} M	(McGuire et al. 1982)
		Reduced	10^{-4} M to 10^{-3} M	(Emonds-Alt et al. 1985)
	Pamidronate	Increased	2.5×10^{-6} M	(Evequoz et al. 1985)
		Increased	10^{-5} M	(Couchman and Sheppard 1986)
	Clodronate	Reduced	2.5×10^{-4} M	(McGuire et al. 1982)
	Tiludronate	Reduced	10^{-5} M to 5×10^{-4} M	(Emonds-Alt et al. 1985);
MMP activity	Zoledronate	Inhibited activity of MMPs-3, 8, 13 and 20	2.5×10^{-5} M to 10^{-3} M	(Heikkila et al. 2002)
		Inhibited activity of MMPs-3, 12, 13 and 20	IC_{50} between 5×10^{-5} M and 1.5×10^{-4} M	(Teronen et al. 1999)
	Pamidronate	Inhibited activity of MMPs-3, 8 and 13	2×10^{-5} M to 10^{-3} M	(Heikkila et al. 2002)
	Alendronate	Inhibited activity of MMPs-3, 8 and 13	2×10^{-5} M to 10^{-3} M	(Heikkila et al. 2002)
		Inhibited activity of MMP-13	IC_{50} between 5×10^{-4} M and 7.5×10^{-4} M	(Kontinen et al. 1999)
	Clodronate	Inhibited activity of MMPs-1 and 8	IC_{50} 1.5×10^{-4} M	(Teronen et al. 1997a; Teronen et al. 1997b)

GAG= glycosaminoglycan; MMP= matrix metalloproteinase; IC_{50} = concentration producing 50% inhibition of activity

in OA that compromises the physio-chemical behaviour of the tissue (Heinegård et al. 2003; Mow and Hung 2003). This compositional change is thought to initiate a series of events that leads to irreversible tissue degradation (Sandy 2006). Thus, reducing the loss of cartilage aggrecan should conserve tissue

integrity and function, and may represent one way of retarding the OA disease process. Since cartilage proteoglycan content is dependent on balance between anabolism and catabolism in matrix (Nagase and Kashiwagi 2003), interventions to increase proteoglycan synthesis or decrease its degradation during the OA process are likely to be beneficial.

At the biochemical level, there is evidence to suggest that zoledronate can reduce cartilage matrix degradation. Zoledronate and other bisphosphonates have been found to inhibit the activity of various MMPs (summarised in Table 1.9), one family of proteinases implicated in cartilage matrix degradation. At the cell/tissue level, bisphosphonates other than zoledronate have been shown to influence chondrocyte viability, matrix synthesis and proteinase production, though both beneficial and detrimental effects have been reported (summarised in Table 1.9). It is not known whether zoledronate has direct effects on cartilage tissue or chondrocytes that alter proteoglycan metabolism.

The following chapters describe studies that have explored hypotheses relating to effects of zoledronate on hyaline cartilage at the cell/tissue level. The general hypothesis was that zoledronate modifies cartilage metabolism to reduce the loss of cartilage glycosaminoglycan during the osteoarthritic process.

Chapter 2. Hypothesis, experimental design and materials and methods

This chapter describes the experimental approach that was taken. Background is provided on the in vitro culture models of OA that were used in the experiments. In addition, the basic materials and methods for the models are detailed.

2.1 General hypothesis

Zoledronate modifies cartilage metabolism to reduce loss of cartilage glycosaminoglycan during the osteoarthritic process

2.2 Experimental design and study objectives

An in vitro approach to investigation was taken to test the general hypothesis since cartilage and chondrocyte metabolism can be studied isolated from other joint tissues. Information was sought by testing individual specific hypotheses in experiments using in vitro models of chondrocyte, chondrocyte-matrix or cartilage metabolism. IL-1, a cytokine involved in regulating cartilage catabolism (discussed in section 1.2.3.6), was employed to stimulate “OA-like” metabolic changes.

Study objectives

- i) Define the concentration range of zoledronate to evaluate in the studies.
- ii) Evaluate zoledronate for effects on GAG synthesis and degradation using culture models of chondrocyte and cartilage metabolism with IL-1 as an OA stimulus.

2.2.1 In vitro models of OA cartilage and OA chondrocyte metabolism

In vitro culture models have been widely used to examine metabolic processes in OA (Caterson et al. 2000). Two model systems were used in this present study. In both, IL-1 was employed as a stimulus of OA metabolic change.

2.2.1.1 Bovine articular cartilage explant culture model

Articular cartilage explants are ex vivo pieces of tissue. When cultured in vitro, the chondrocytes within the explants are maintained in their usual three-dimensional matrix. Bovine articular cartilage is often used in this culture model and the addition of the catabolic cytokine IL-1 stimulates proteoglycan degradation that can be measured as released GAG or aggrecan in the culture medium (Caterson et al. 2000; Little et al. 1999; Sandy et al. 1991a; Sandy et al. 1991b). Western blot analysis of the medium using monoclonal antibodies to identify cleavage sites on released aggrecan fragments indicates that aggrecanase activity is primarily responsible for aggrecan catabolism in this culture system. Furthermore, IL-1 up-regulates aggrecanase-1 and aggrecanase-2 mRNA transcription in bovine cartilage explants. Taken together, the metabolic events that characterise the model simulate the cartilage metabolic processes in vivo which lead to the loss of cartilage GAG during OA pathogenesis (discussed in section 1.2.3.3).

2.2.1.2 Chondrocyte/ alginate bead culture model

Chondrocyte and matrix metabolism may also be studied in a three-dimensional culture system in which cells that have been isolated from cartilage are cultured encapsulated and suspended in alginate gel beads. Alginates, glycuranans extracted from brown seaweed algae (Guo et al. 1989), are linear unbranched polymers containing D-mannuronic acid and L-guluronic acid residues that form gels in the presence of calcium or other multivalent counter ions (Chaplin 2007).

The morphologic and metabolic features of this culture system have been extensively characterised, indicating that cells retain their chondrocyte phenotype. The cells are observed (i) to maintain a spherical shape (Guo et al. 1989); (ii) to synthesise characteristic matrix molecules (aggrecan and type II collagen) (Almqvist et al. 2001; Hauselmann et al. 1992; Hauselmann et al. 1994); and (iii) to express MMPs (Chubinskaya et al. 2001). These characteristics appear to persist for some time and even after 8 months of culture, the cells remain metabolically active and continue to synthesise type II collagen and aggrecan (Hauselmann et al. 1994).

The distribution and composition of elaborated matrix in the culture model also demonstrates similarities to that found in articular cartilage. Long-term culture of normal adult human chondrocytes in alginate beads establishes matrix that is found in two compartments: the cell-associated and further-removed matrix. These compartments correspond respectively to the pericellular/territorial and interterritorial matrix of articular cartilage (Hauselmann et al. 1996b). By day 30 of culture, the absolute and relative volumes occupied by these compartments and the encapsulated cells were observed to be nearly identical to those found in native articular cartilage. Furthermore, other investigators have shown that after 4 weeks of culture, collagen types II, IX and XI are seen to accumulate in the matrix in relative proportions that are similar to those found in adult cartilage (Petit et al. 1996).

Collectively, these observed cell and matrix characteristics indicate that the chondrocyte phenotype is maintained in the chondrocyte/ alginate culture system. Thus, as a model for studying chondrocyte metabolism it has advantages over the two-dimensional culture of chondrocytes in monolayer, a model that suffers from problems with chondrocyte dedifferentiation and loss of phenotypic traits (Benya and Shaffer 1982; Holtzer et al. 1960). Additionally, in comparison to cartilage explant culture, the chondrocyte/ alginate culture technique allows intra-joint topographical variability in metabolism to be somewhat minimalised through the removal of native cartilage matrix and the

pooling of isolated chondrocytes, the benefit being that small-sized treatment effects on cell metabolism should be more easily observed.

Effects of IL-1 on cell and matrix metabolism in the chondrocyte/ alginate bead culture model

In the alginate bead culture model, IL-1 has been found to have inhibitory effects on proteoglycan synthesis by bovine articular chondrocytes (D'Souza et al. 2000) and aggrecan production by human OA chondrocytes (Sanchez et al. 2002). Effects on matrix degradation have also been seen. Following the culture of bovine articular chondrocytes in alginate beads for two weeks to establish matrix, subsequent treatment with IL-1 induced profound GAG release from the beads into the media (Beekman et al. 1998). Though the exact mechanism for GAG release induced by IL-1 in the model was not demonstrated specifically, MMP activity was not increased implying that other proteolytic enzymes such as aggrecanases might have been responsible for GAG catabolism.

Thus, the chondrocyte/ alginate bead culture model has compositional and metabolic characteristics that mimic native cartilage and addition of IL-1 to the culture system allows aggrecan catabolism to be investigated. Previously, several studies have used this model to investigate the effects of agents on chondrocyte metabolism to help define therapeutic potential in OA (Henrotin et al. 2003; Sanchez et al. 2002; Sanchez et al. 2003).

2.2.1.3 Form and concentration of IL-1 used in the in vitro studies

IL-1 is the prototypic pro-inflammatory cytokine and is found in two forms, IL-1 α and IL-1 β (Dinarello 1997). In most studies both forms have very similar biological activities, however, bovine articular chondrocytes appear more sensitive to human recombinant IL-1 α than to IL-1 β . IL-1 α has been found to have a greater inhibitory effect than IL-1 β on proteoglycan synthesis by bovine articular chondrocytes cultured in agarose gel and only IL-1 α was capable of stimulating proteoglycan degradation (Aydelotte et al. 1992). In addition, IL-1 α is

the form of the cytokine that has been frequently used as a catabolic stimulus to study aggrecan degradation in bovine articular cartilage explants (Little et al. 1999; Sandy et al. 1991a; Sandy et al. 1991b). Thus, for the culture experiments of this present study, IL-1 α was selected as the form of the cytokine to be used to stimulate proteoglycan degradation and inhibit proteoglycan synthesis.

The concentration of IL-1 α chosen for the majority of experiments was 10 ng/ml, based on upon a previously described method for bovine articular cartilage explant culture (Little et al. 1999). Though for the purposes of in vitro investigation with short culture periods, 10 ng/ml IL-1 α is an effective stimulus of cartilage proteoglycan degradation, it is important to note that in vivo the synovial fluid concentrations of IL-1 (albeit IL-1 β rather than IL-1 α) from patients with knee OA are reported to be nearly 100-fold lower (Westacott et al. 1990). The difference is striking but might be partly explained by interactions of various cytokines in vivo, such as TNF α acting synergistically with IL-1 to drive cartilage destruction (Goldring 2000; Goldring and Goldring 2004). Furthermore, another difference is that in vitro the cytokine is employed to stimulate cartilage degradation over a relatively short time period (e.g. 4 days), whereas in vivo this process is much more prolonged.

2.2.2 Use of culture models to fulfil study objectives

Study objective (i). Define the concentration range of zoledronate to evaluate in the studies.

Experiments using the bovine chondrocyte/ alginate bead culture model were performed to investigate adverse effects of zoledronate on chondrocytes to define the upper limit of the concentration range. Specific outcomes measured were cell viability, proliferation and GAG synthesis. The results on GAG synthesis served partly to deliver study objective (ii).

Study objective (ii). Evaluate zoledronate for effects on GAG synthesis and degradation using culture models of chondrocyte and cartilage metabolism with IL-1 as an OA stimulus.

Both the bovine cartilage explant culture model and the bovine chondrocyte/alginate bead culture model were used. The outcome measured was the amount of GAG released from the tissue.

2.3 Materials and methods

2.3.1 Basic methods: cell and tissue culture

Tissue source

Bovine forefeet were obtained on the day of animal slaughter from a local abattoir and used within 24 hours. All animals were aged 12-30 months and of unknown breed and sex unless otherwise specified.

2.3.1.1 Culture of bovine articular cartilage explants

Medium and general maintenance conditions

The basic medium used for all explant cultures was Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, UK) supplemented with 50 µg/ml gentamicin (Gibco) and 0.5% (v/v) antibiotic/antimycotic solution (100x containing 10,000 units penicillin, 10,000 µg streptomycin, and 25 µg amphotericin B/ml; Gibco). All explant cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Preparation and culture of explants

Bovine articular cartilage explant culture was performed according to previously described methods (Little et al. 1999). Only one joint from one foot was used for each experiment to exclude inter-animal and inter-joint variation. Fore feet were washed and the skin was excised. After exposing metacarpophalangeal (MCP) joints under aseptic conditions, full depth articular cartilage slices were excised off the metacarpal articular surface with a scalpel (approximately 80 explants per articular surface), pooled, washed with DMEM and then pre-cultured in DMEM supplemented with 10% (v/v) foetal calf serum (FCS; First Link, West Midlands, UK) for 48 hours. Explants were then washed 3 times for 10 minutes each in serum-free DMEM before treatment culture. Methods for treatment culture are detailed in Chapter 4.

2.3.1.2 Culture of cells isolated from bovine articular cartilage in alginate beads

Medium and general maintenance conditions

The basic medium used for all cell cultures was DMEM supplemented with 150 mg/ml ascorbic acid (Sigma, Poole, UK), 100 unit/ml penicillin (Gibco) and 100 µg/ml streptomycin. All cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Isolation of cells

Articular cartilage was obtained using the same method as for explant culture. Cartilage slices obtained from one to seven MCP joints were pooled, washed with Earle's balanced salt solution (EBSS; Gibco) and cultured overnight in DMEM supplemented with 20% FCS. The next day the cartilage was finely diced with a scalpel and then, to digest the matrix and release cells, the tissue was incubated for 1 hour at 37°C in DMEM + 20% FCS and 10 mg/ml pronase E (BDH, Lutterworth, Leics, UK) and then for further 16 hours at 37°C in DMEM + 20% FCS and 100 unit/ml collagenase XI (Sigma). The resulting cell

suspension was passed through a 70 µm pore size sieve (Falcon, Oxford, UK) and washed twice with EBSS. Viability and total cell numbers were determined using the trypan blue (Sigma) exclusion method and a cell counting chamber. Cell viability ranged from 96% to 100%. Cells were resuspended in DMEM + 20% FCS to yield a suspension with 2×10^7 cells/ml.

Forming alginate gel beads containing cells

Alginate Keltone LV (Kelco Nutrasweet, Poole, UK) was dissolved in EBSS and autoclaved to give a sterile 4% (w/v) alginate solution which was added to an equal volume of the cell suspension to yield a final concentration of 2% alginate containing 1×10^7 cells/ml. The cell/alginate suspension was slowly expressed through a 25-gauge needle into a solution containing 100 mM CaCl₂ (BDH) in phosphate buffered saline (PBS; Sigma) to form alginate beads containing the cells. The bead volume and cells per bead were calculated. Beads were incubated at room temperature in the CaCl₂/PBS solution for 10 mins to induce crosslinking of the alginate gel and then washed three times with EBSS followed by a final wash with DMEM + 20% FCS. Different methods were used for the subsequent culture of the beads and these are detailed in the materials and methods section of each experimental chapter.

2.3.2 Basic methods: statistical analyses

Statistical analyses were performed using SPSS 14.0 for Windows software (SPSS Inc.). Assumptions about the normal distribution and homogeneity of variance in relation to the data were assessed by frequency plots and Levene's test respectively. Because nearly all data sets fulfilled these assumptions, all comparisons were carried out using parametric tests to maintain consistency. First, data were analysed by ANOVA and if differences of the means between groups were found ($P < 0.05$), post-hoc comparisons were then performed. Within experiments, comparisons of means between two groups were made using the Independent-Samples T Test and comparisons of means between multiple groups were made with Dunnett's t test or the Bonferroni test. When

experimental hypotheses were tested in a single experiment, the alpha level for statistical significance between means was set at 0.05. When multiple experiments were performed to test the same hypothesis, a more stringent alpha level of 0.01 was used in order to take into account the increased likelihood of statistical type 1 error. In the event that data sets showed a non-normal distribution or exhibited unequal variance, any comparisons with significance levels near to the set alpha level were interpreted cautiously.

Chapter 3. Effects of zoledronate on bovine articular chondrocyte viability, proliferation and proteoglycan synthesis. Selection of the zoledronate concentration range to evaluate.

There were two aims behind the experiments described in this chapter. In order to aid selection of the concentration range to evaluate in further studies, the adverse effects of zoledronate on chondrocyte metabolism (cell viability, proliferation and proteoglycan synthesis) were investigated. In addition, another purpose for examining effects on chondrocyte proteoglycan synthesis was to provide information for the general hypothesis.

3.1 Introduction

Three criteria were considered in relation to the concentration range of zoledronate to evaluate for effects on cartilage or chondrocyte proteoglycan metabolism. Ideally, the range should include zoledronate concentrations that were achieved in cartilage in the *in vivo* studies that found beneficial treatment effects with respect to cartilage outcomes. In addition, zoledronate concentrations with known *in vitro* cellular effects or inhibitory MMP activities should be covered and, moreover, the upper limit of the range should be defined by zoledronate concentrations that have adverse effects on chondrocytes.

Reduction in cartilage damage in rabbits was seen with subcutaneous zoledronate 10 µg/kg given three times a week (Muehleman et al. 2002), a dosing regimen that approximates to intravenous 4 mg zoledronate given monthly in humans (personal communication from Dr. J.R. Green, Novartis Pharma AG). Patients with Paget's disease, in whom zoledronate treatment reduced a urinary marker of type II collagen degradation (CTX II), received either a single 200 µg or 400 µg intravenous dose of zoledronate (Garnero et al. 2001a).

It is not possible to make accurate estimates for zoledronate cartilage tissue concentrations achieved in either of the above studies since specific pharmacokinetic distribution data for cartilage have not been reported. Available data indicate that in humans receiving a single dose of intravenous zoledronate 4 mg, the mean peak plasma concentration was 309 ng/ml (about 10^{-6} M) which then declined rapidly to <1% of the peak twenty-four hours post infusion (Skerjanec et al. 2003). Extrapolating from these data, the 400 µg dose of zoledronate should have lead to an approximate peak plasma concentration of 10^{-7} M in the Paget's disease patients. Drug distribution data have also been reported for systemically-administered zoledronate in rats (Green and Rogers 2002). In rat bone, zoledronate was found in high concentrations between 10^{-4} mol/kg and 10^{-3} mol/ kg, levels which persisted up to eight months after injection. During this period, zoledronate was present also in soft tissue (10^{-7} mol/kg to 10^{-6} mol/kg) and blood (10^{-9} mol/kg to 10^{-7} mol/kg). Despite the lack of reports to indicate that systemically-administered zoledronate can distribute to cartilage, there are theoretical mechanisms by which the bisphosphonate could reach cartilage, namely via subchondral bone or the circulation (discussed in 1.3.3.2).

Numerous in vitro studies have found effects of zoledronate on various enzyme, cell and tissue metabolic processes (Table 3.1). These effects were observed at zoledronate concentrations between 2×10^{-9} M to 10^{-3} M and, in particular, cell toxicity was demonstrated in rabbit osteoclasts (Benford et al. 2001), human foetal osteoblasts (Reinholz et al. 2000) and human myeloma cells (Derenne et al. 1999) at concentrations 10^{-5} M and above. In addition, other in vitro studies have found that etidronate, clodronate, pamidronate and risedronate have detrimental effects on chondrocytes, mostly with bisphosphonate concentrations around the 10^{-4} M mark (Guenther et al. 1979; Palmoski and Brandt 1978; Van Offel et al. 2002). However, it is not known whether zoledronate has adverse effects on chondrocyte metabolism.

Table 3.1. Some reported effects of zoledronate on various metabolic processes that have been observed in vitro

Effect on metabolic process	Zoledronate concentration for observed effect	Reference
Bone-related		
Inhibition of protein prenylation in rabbit osteoclasts	10^{-5} M	(Coxon et al. 2000)
Inhibition of recombinant human farnesyl diphosphate synthase activity	3×10^{-9} M (IC_{50})	(Dunford et al. 2001)
Stimulation of caspase-3-like activity in rabbit osteoclasts	10^{-4} M	(Benford et al. 2001)
Inhibition of 1,25-dihydroxyvitamin D ₃ -induced bone resorption in mouse calvaria	2×10^{-9} M (IC_{50})	(Green et al. 1994)
Inhibition of calcium incorporation into calvaria	10^{-5} M to 10^{-4} M	(Green and Rogers 2002)
Inhibition of human foetal osteoblast proliferation	10^{-5} M to 10^{-4} M	(Reinholz et al. 2000)
Stimulation of human foetal osteoblast mineralisation of matrix	10^{-5} M	
MMP-related		
Inhibition of MMP-20 activity against β -casein	5×10^{-5} M to 5×10^{-4} M	(Heikkila et al. 2002)
Inhibition of MMP-3, MMP-8 and MMP-13 activity against β -casein	2×10^{-5} M to 10^{-3} M	
Inhibition of MMP-1 production by bone marrow stromal cells stimulated by IL-1 β	10^{-6} M	(Derenne et al. 1999)
Stimulation of MMP-2 secretion by bone marrow stromal cells	10^{-9} M to 10^{-6} M	
Myeloma-related		
Inhibition of human myeloma cell proliferation	5×10^{-5} M to 5×10^{-4} M	(Derenne et al. 1999)
Stimulation of bone marrow stromal cell apoptosis	10^{-4} M	
Stimulation of human myeloma cell apoptosis	10^{-4} M to 5×10^{-4} M	

IC_{50} = concentration producing 50% inhibitory effect

On the other hand, zoledronate may have beneficial effects on chondrocyte metabolism. In relation to the general hypothesis, one way that zoledronate could improve the proteoglycan content in OA cartilage is by increasing chondrocyte proteoglycan synthesis. Interestingly, clodronate (2.5×10^{-5} M and 2.5×10^{-4} M) has been found to stimulate GAG synthesis in rabbit articular chondrocytes (Guenther et al. 1979), though another study found that both etidronate and clodronate (10^{-6} M to 5×10^{-4} M) inhibited GAG synthesis in canine articular cartilage explants (Palmoski and Brandt 1978).

It is not known (i) whether zoledronate has adverse effects on chondrocyte metabolism and (ii) whether the bisphosphonate can stimulate chondrocyte proteoglycan synthesis. The experiments described in this chapter have been performed to determine this information using the bovine articular chondrocyte/alginate bead culture model with and without IL-1 α co-treatment as an OA stimulus (model described in section 2.2.1).

3.2 Experimental hypotheses

Zoledronate at concentrations 10^{-5} M and above reduces cell viability and inhibits cell proliferation and proteoglycan synthesis of bovine articular chondrocytes cultured in alginate beads with and without IL-1 α co-treatment

Zoledronate at concentrations without adverse effects on cell viability or proliferation stimulates the proteoglycan synthesis of bovine articular chondrocytes cultured in alginate beads with and without IL-1 α co-treatment

3.3 Experimental objectives

- i)** Culture bovine articular chondrocytes in alginate beads with and without zoledronate (10^{-12} M to 10^{-4} M) and with and without IL-1 α (10 ng/ml)
- ii)** Measure cell viability
- iii)** Measure rates of cell proliferation by 3 H-TdR incorporation
- iv)** Measure rates of cell proteoglycan synthesis by 35 SO₄ incorporation

3.4 Materials and methods

3.4.1 Cell culture and treatments

Bovine articular chondrocyte/ alginate bead culture

Alginate beads containing cells isolated from bovine articular cartilage were formed as described in section 2.3.1.2. Four experiments assessing cell viability (exps. b.a2, b.a4.1, b.a5.1 and b.a6.2) were performed on separate occasions using different tissue sources. Another experiment was performed to assess cell proliferation and proteoglycan synthesis (exp. b.a6.1) using the same tissue source as experiment b.a6.2.

Differences in the culture method and outcome measure between experiments are summarised in Table 3.2. Experiment b.a2: treatment commenced on the day of bead formation. Experiments b.a4.1, b.a5.1, b.a6.1 and b.a6.2: beads were pre-cultured in DMEM + 20% FCS for 3 days in 225 cm² tissue culture flasks (Corning Life Sciences, Schipol-Rijk, The Netherlands) prior to commencing treatment culture. This 3-day pre-culture period allows stabilisation of the cells in alginate as previously described (van Susante et al. 2000).

Beads were then washed 3 times for 10 minutes each in DMEM. Up to 6 beads were placed in individual wells of 24-well tissue culture plates. Into each well were added 1 ml of treatment serum-free DMEM (experiments b.a4.1 and b.a5.1) or DMEM + 10% FCS (experiments b.a2, b.a6.1 and b.a6.2) with and

without 10 ng/ml IL-1 α and with and without zoledronate treatment concentrations. For experiment b.a6.1 treatment DMEM was supplemented also with 37 kBq/ml tritiated thymidine (^3H -TdR; Amersham Biosciences UK, Bucks, UK) and 370 kBq/ml $^{35}\text{SO}_4$ (Amersham Biosciences UK). Treatment cultures were performed in replicates of 6 and maintained in treatment DMEM for 1 or 2 days. Cell viability was assessed after 1 or 2 days culture. For experiment b.a6.1 beads and medium were harvested after 2 days of culture and stored separately at -20°C until biochemical analysis.

3.4.2 Cell viability analysis

Beads were washed twice with PBS. Cell viability was assessed using the Live/dead viability/cytotoxicity kit (Molecular Probes Inc., Eugene, Oregon, USA). This technique distinguishes live cells by the presence of ubiquitous intracellular esterase activity that enzymatically converts virtually non-fluorescent cell-permeant calcein AM to calcein producing intense green fluorescence. Dead cells with damaged membranes allow ethidium homodimer-1 (EthD-1) to enter and bind to nucleic acids to produce bright red fluorescence. Beads were incubated for 1 hour at 37°C in 0.5 ml of PBS containing 4 μM EthD-1 and 1 μM calcein AM. Each bead was sliced, placed on a glass slide, covered with a coverslip, viewed by fluorescence microscopy and photographed (Fig 3.2). Live cells and dead cells were manually counted within a defined area and cell viability was calculated as follows:

$$\% \text{ cell viability} = \frac{\text{number live cells}}{\text{number live cells} + \text{number dead cells}} \times 100$$

Reproducibility of manual cell counting: coefficient of variation (CV) < 1% for counts repeated 3 times.

Table 3.2. Summary of cell source characteristics and culture conditions for experiments evaluating the effects of zoledronate on cell viability, proliferation and proteoglycan synthesis in the bovine chondrocyte/ alginate bead culture model.

Exp.	Characteristics of cell source	Cell viability post isolation	Bead volume; cells/ bead; beads/ well	Pre-culture; medium	No. days of treatment culture; medium	Treatment	Outcome measured
b.a2	1x MCP joint from bovine animal aged 12-30 months; sex unknown	96.4%	6.8 µl; 67600; 3	Not performed	1 day; DMEM + 10% FCS	IL-1 α 0, 10 ng/ml; Zol 0, 10^{-12} M to 10^{-4} M	Cell viability
b.a4.1	2x MCP joints from 2 bulls each aged 13 months	100%	7.4 µl; 73500; 3	3 days; DMEM + 20% FCS	1, 2 days; serum-free DMEM	IL-1 α 0, 10 ng/ml; Zol 0, 10^{-9} M to 10^{-6} M	Cell viability
b.a5.1	2x MCP joints from 2 heifers aged 24 and 29 months	100%	N/A; N/A; 6	3 days; DMEM + 20% FCS	2 days; serum-free DMEM	IL-1 α 0, 10 ng/ml; Zol 0, 10^{-8} M to 10^{-4} M	Cell viability
b.a6.1	7x MCP joints from 4 heifers each aged 18 months	99%	7.1 µl; 71400; 6	3 days; DMEM + 20% FCS	2 days; DMEM + 10% FCS	IL-1 α 0, 10 ng/ml; Zol 0, 10^{-10} M to 10^{-4} M	Cell proliferation and proteoglycan synthesis
b.a6.2	7x MCP joints from 4 heifers each aged 18 months	99%	7.1 µl; 71400; 6	3 days; DMEM + 20% FCS	2 days; DMEM + 10% FCS	IL-1 α 0, 10 ng/ml; Zol 0, 10^{-10} M to 10^{-4} M	Cell viability

Exp= experiment; MCP= metacarpophalangeal; DMEM= Dulbecco's modified Eagle's medium; FCS= foetal calf serum; Zol= zoledronate

3.4.3 Biochemical analyses

Alginate beads were disrupted and digested by incubating at 60°C for 24 h in 1ml of 55 mM sodium citrate (BDH), 150 mM sodium chloride (Sigma), 5 mM cysteine hydrochloride, 5 mM EDTA (BDH) and 0.56 units/ml papain (Sigma) as previously described (Enobakhare et al. 1996). All biochemical analyses of samples were performed in duplicate.

Quantification of ^3H -TdR incorporation

Rates of cell proliferation were assessed by quantifying amounts of ^3H -TdR incorporated into newly-formed DNA over the two day incubation period using the trichloroacetic acid (TCA) precipitation technique and liquid scintillation counting. Aliquots (100 μl) of the alginate bead digest were added to individual wells of a multiscreen plate (0.65 μm pore size; Millipore, Watford, Herts, UK) followed by 100 μl of 20% (w/v) TCA (BDH) and incubated at 4°C for 30 min. The multiscreen plate was vacuum aspirated and wells were rinsed twice with 100 μl 10% TCA, vacuum aspirating each time. The plate was dried and the filters were punched out into scintillation vials. Five hundred μl of 0.01M KOH (BDH) was added to each of the vials which were then agitated for 2 h to release bound ^3H -Tdr from the filter into solution. A 4.5 ml volume of Ultima GoldTM MV liquid scintillation cocktail (Perkin Elmer LAS UK, Beaconsfield, Bucks, UK) was added to each vial and scintillations were measured using a Perkin Elmer Tricarb 2900 TR scintillation counter (Perkin Elmer LAS UK). Reproducibility: mean intra-assay CV 1.8%.

Quantification of $^{35}\text{SO}_4$ incorporation

Rates of proteoglycan synthesis were assessed by measuring the amounts of $^{35}\text{SO}_4$ incorporated into newly-synthesised proteoglycans and/or glycosaminoglycans over the two day incubation period using an alcian blue precipitation method and liquid scintillation counting (Masuda et al. 1994). Aliquots (75 μl) of a pH 5.8 solution containing 50 mM sodium acetate (BDH) and 0.5% (v/v) triton X-100 (BDH) were added to individual wells of a multiscreen plate (0.65 μm pore size) followed by 40 μl aliquots of medium or

alginate bead digest and 150 µl of alcian blue solution consisting of 0.2% (w/v) alcian blue 8GX (BDH) in 50 mM sodium acetate, 85 mM magnesium chloride, pH 5.8. The plate was gently mixed for 1 h at room temperature and vacuum aspirated. Wells were washed 3 times with 200 µl volumes of a pH 5.8 solution containing 50 mM sodium acetate, 85 mM magnesium chloride and 100 mM sodium sulphate, vacuum aspirating on each occasion. The filters were punched out into scintillation vials and gently agitated for 1 h in 0.5 ml of 4 M guanidine HCl in 33% (v/v) propan-2-ol. Four ml of Ultima GoldTM MV was added to each vial and scintillations measured using a Perkin Elmer Tricarb 2900 TR scintillation counter. Reproducibility: mean intra-assay CV 1.9%.

DNA quantification

Amounts of DNA contained in the beads were determined using the Hoechst fluorimetric method (Rao and Otto 1992). Aliquots (100 µl) of standards prepared using calf thymus DNA (Sigma) in pH 7.0 saline sodium citrate (SSC; 150 mM NaCl and 15 mM sodium citrate; both from BDH) or alginate bead digests were placed in individual wells of 96-well microtitre plates (Nunc, Roskilde, Denmark). Aliquots (100 µl) of 2 µg/ml Hoechst 33258 (Sigma) in SSC were added to each well and then the plate was read using a Fluoroskan Ascent microtitre fluorimeter (Labsystems Oy, Helsinki, Finland) with excitation at 348 nm and emission readings at 460 nm. Values for the DNA content of the alginate beads were used to normalise values obtained for sulphate incorporation. Reproducibility: mean intra-assay CV 1.8%

3.4.4 Statistical analyses

Statistical analyses were performed as described in section 2.3.2. With respect to % cell viability data, phrases such as “caused a 10% reduction” or “decreased by 10 %” are used in relation to absolute differences in values. With respect to cell proliferation and proteoglycan synthesis data, these phrases indicate the proportional differences between values.

3.5 Results

3.5.1 Effects of zoledronate on chondrocyte viability with and without IL-1 α co-treatment

Four experiments were performed to assess the effects of zoledronate on chondrocyte viability with and without IL-1 α co-treatment (experiments b.a2, b.a4.1, b.a5.1 and b.a6.2).

Chondrocyte viability in controls and IL-1 α -treated controls (Table 3.3)

In controls, with or without IL-1 α 10 ng/ml, cell viabilities after one-day or two-day culture ranged from 92.1% to 98.2%. IL-1 α 10 ng/ml did not affect cell viability in the one-day cultures, but in cultures for two days caused a 3.1% reduction in cell viability compared to no cytokine in one experiment ($P<0.001$) and a trend for a 1.7% reduction in another ($P=0.049$). Duration of culture also had an effect. Comparing two-day culture to one-day culture in the single experiment in which both time points were examined (exp. b.a4.1), a trend for a 1.5% reduction in cell viability was observed in controls ($P=0.049$) and a 2.7% reduction was seen in IL-1 α -treated controls ($P=0.001$).

Table 3.3. Cell viability after one-day or two-day culture of bovine articular chondrocytes in alginate beads in controls and IL-1 α -treated controls.

Culture period	Experiment	Mean % cell viability		P value for difference in means between control and IL-1 α -treated control*
		Control	IL-1 α -treated control	
One day of culture	b.a2	94.4	95.2	$P=0.413$
	b.a4.1	98.2	97.7	$P=0.118$
Two days of culture	b.a4.1	96.7	95.0	$P=0.049$
	b.a5.1	92.6	93.1	$P=0.555$
	b.a6.2	95.2	92.1	$P=0.000183^{**}$

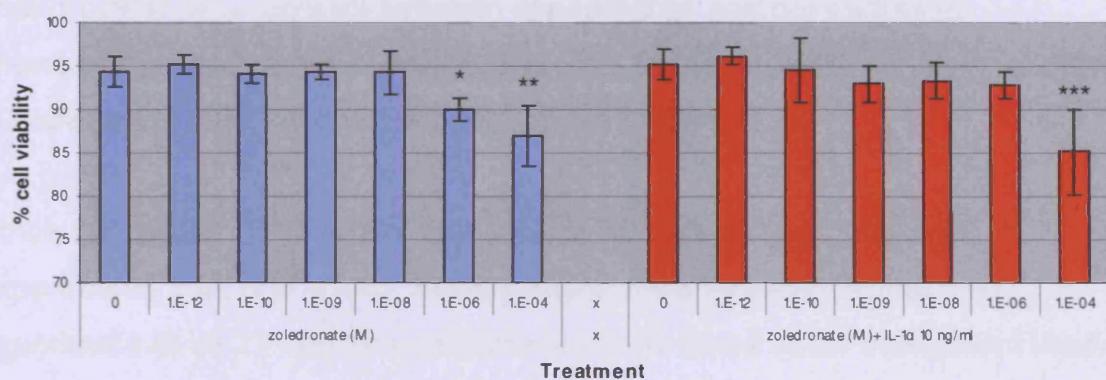
* Since multiple experiments were performed the alpha level to indicate a significant difference between means was set at $P=0.01$ as described in section 2.3.2.

** Statistically significant difference at the $P<0.01$ level.

Effects of zoledronate on chondrocyte viability

In the first experiment (b.a2), one-day treatment cultures were carried out. In basal cultures (i.e. without IL-1 α co-treatment), zoledronate 10 $^{-6}$ M and zoledronate 10 $^{-4}$ M reduced cell viability compared to control by 4.3% (P=0.002) and 7.4% (P<0.001) respectively (Fig. 3.1; Table 3.4). Fig. 3.2 shows photographs of the cells following treatment for control and zoledronate 10 $^{-4}$ M. In IL-1 α co-treated cultures, zoledronate 10 $^{-4}$ M caused a 10.1% reduction in cell viability (P<0.001; Fig. 3.1; Table 3.4). Lower concentrations of zoledronate down to 10 $^{-12}$ M did not affect cell viability in either basal or IL-1 α co-treated cultures.

Fig. 3.1. Experiment b.a2: Effects of zoledronate (1.E-12 M to 1.E-4 M) with and without IL-1 α co-treatment on the viability of chondrocytes cultured in alginate beads after one-day treatment culture.



Bovine articular chondrocytes cultured in alginate beads in the presence of treatments for one day prior to assessment of chondrocyte viability. Values for %cell viability shown as means \pm SD (n=6). *P=0.002 or **P<0.001 vs. zoledronate 0M. ***P<0.001 vs. zoledronate 0M + IL-1 α 10 ng/ml.

As zoledronate 10 $^{-6}$ M and zoledronate 10 $^{-4}$ M had been observed to reduce cell viability in experiment b.a2 but concentrations between 10 $^{-8}$ M and 10 $^{-6}$ M had not been explored, an additional experiment (b.a4.1) was performed to determine adverse effects of zoledronate 10 $^{-9}$ M to 10 $^{-6}$ M (x10 increments between concentrations). However, no zoledronate treatment effects on cell viability were seen in one-day or two-day cultures with or without

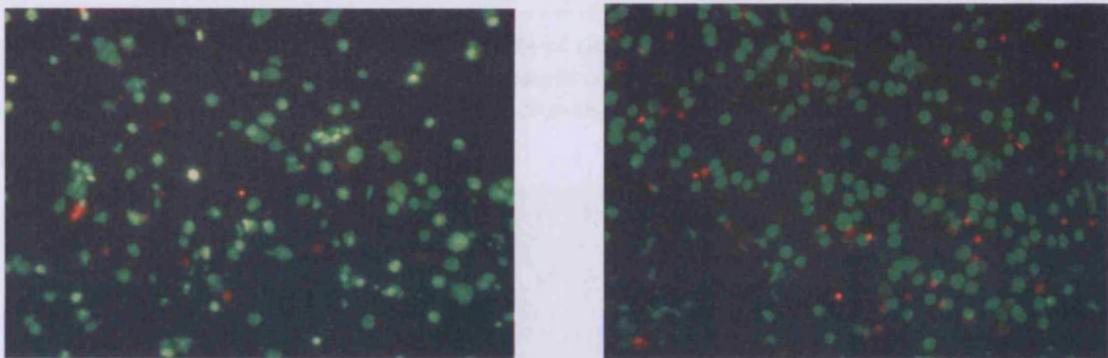
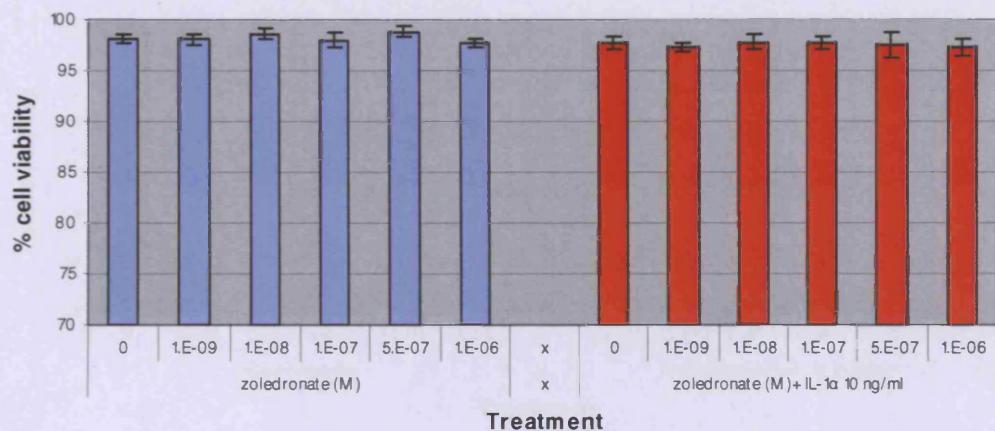


Fig 3.2. Experiment b.a2: Photographs of chondrocytes after live/dead staining.
Examples of photographs taken through a fluorescent microscope after one day of treatment culture and subsequent incubation in live/dead stain (live cells appear green and dead cells red); cells counted in photographs to measure %cell viability (results detailed elsewhere). Left: control culture; right: culture with zoledronate 10^{-4} M treatment.

IL-1 α co-treatment (Figs 3.3 and 3.4; Table 3.4). Of note, the animal source for chondrocytes was different between experiments, and pre-culture of chondrocytes and serum supplementation of treatment medium were performed in experiment b.a4.1 but not experiment b.a2 (Table 3.2).

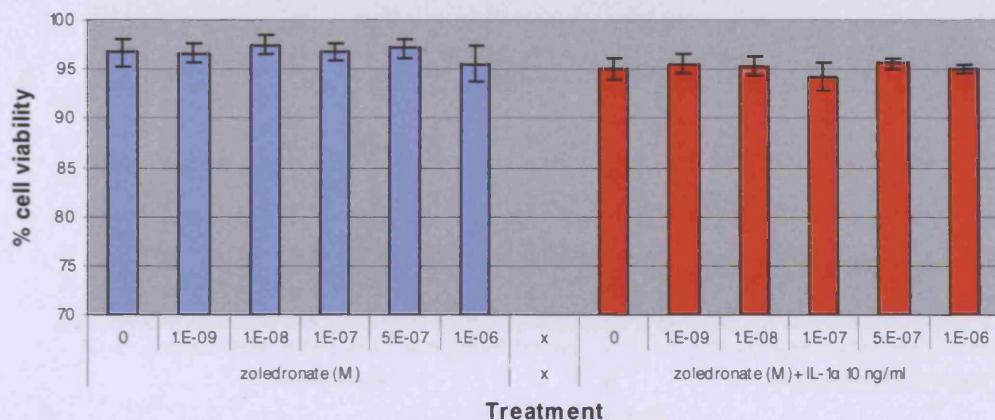
Since the results for effects of zoledronate 10^{-6} M disagreed between experiments b.a2 and b.a4.1, investigations were repeated. In the next experiment (b.a5.1), culture conditions were the same as for experiment b.a4.1 except chondrocytes were sourced from 24- to 29-month old heifers instead of 13-month old bulls and only two-day culture was performed (Table 3.2). The concentration range investigated was extended back up to 10^{-4} M. Compared to control, zoledronate 10^{-4} M reduced cell viability by 2.6% in basal cultures ($P=0.003$; Fig. 3.5; Table 3.4). No other effects were observed for lower zoledronate concentrations in basal cultures or for any concentration (10^{-8} M to 10^{-4} M) in IL-1 α co-treated cultures.

Fig. 3.3. Experiment b.a4.1: Effects of zoledronate (1.E-9 M to 1.E-6 M) with and without IL-1 α co-treatment on the viability of chondrocytes cultured in alginate beads after one-day treatment culture.



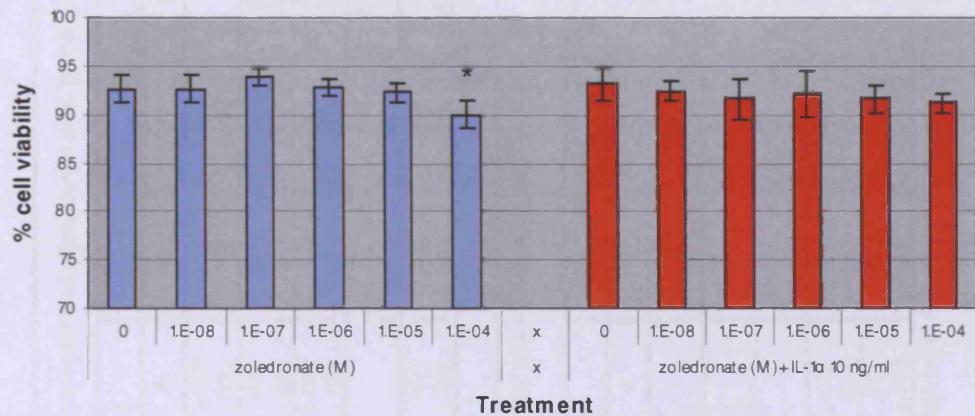
Bovine articular chondrocytes cultured in alginate beads in the presence of treatments for one day prior to assessment of chondrocyte viability. Values for % cell viability shown as means \pm SD (n=6).

Fig. 3.4. Experiment b.a4.1: Effects of zoledronate (1.E-9 M to 1.E-6 M) with and without IL-1 α co-treatment on the viability of chondrocytes cultured in alginate beads after two-day treatment culture



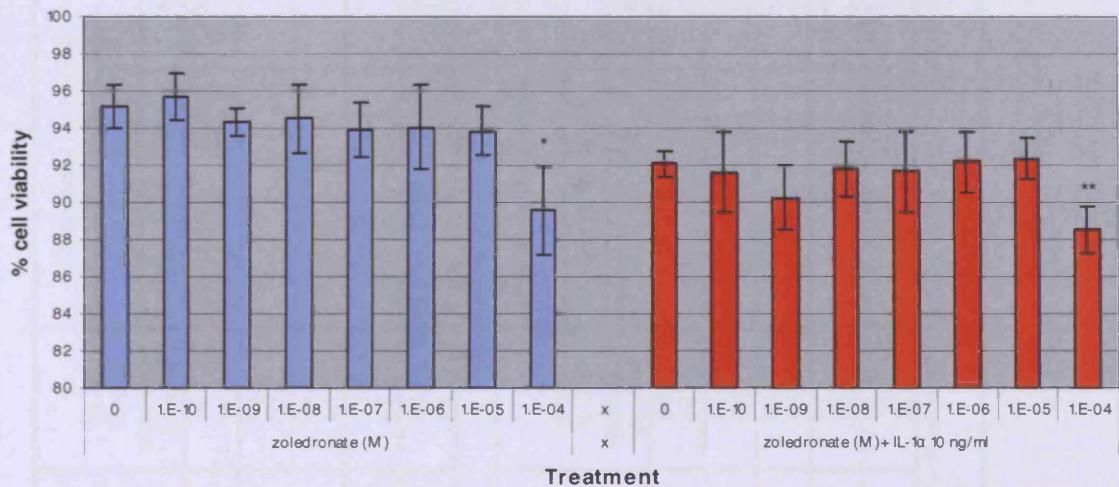
Bovine articular chondrocytes cultured in alginate beads in the presence of treatments for two days prior to assessment of chondrocyte viability. Values for % cell viability shown as means \pm SD (n=6).

Fig. 3.5. Experiment b.a5.1: Effects of zoledronate (1.E-8 M to 1.E-4 M) with and without IL-1 α co-treatment on the viability of chondrocytes cultured in alginate beads after two-day treatment culture



Bovine articular chondrocytes cultured in alginate beads in the presence of treatments for two days prior to assessment of chondrocyte viability. Values for % cell viability shown as means \pm SD (n=6). *P=0.003 vs. zoledronate 0M.

Fig. 3.6. Experiment b.a6.2: Effects of zoledronate (1.E-10 M to 1.E-4 M) with and without IL-1 α co-treatment on the viability of chondrocytes cultured in alginate beads after two day treatment culture.



Bovine articular chondrocytes cultured in alginate beads in the presence of treatments for two days prior to assessment of chondrocyte viability. Values for % cell viability shown as means \pm SD (n=6). *P<0.001 vs. zoledronate 0M. **P=0.003 vs. zoledronate 0M + IL-1 α 10 ng/ml.

Table 3.4. Results summary from four experiments for effects of zoledronate (1.E-12 M to 1.E-4 M) with and without IL-1 α co-treatment on chondrocyte viability after one-day or two-day treatment culture

Exp.	Treatment culture duration (days)	IL-1 α co-treatment (ng/ml)	Mean % cell viability of control	Difference (Δ) in mean % cell viability between control and treatment and significance level of the difference (P*)									
				Zol 10 ⁻¹² M		Zol 10 ⁻¹⁰ M		Zol 10 ⁻⁹ M		Zol 10 ⁻⁸ M		Zol 10 ⁻⁷ M	
				Δ	P	Δ	P	Δ	P	Δ	P	Δ	P
b.a2	1	0	94.4	+0.8	.934	-0.2	>.999	-0.1	>.999	-0.1	>.999		
		10	94.8	+0.9	.980	-0.7	.995	-2.3	.523	-1.9	.688		
b.a4.1	1	0	98.2					-0.1	.992	+0.5	.362	-0.2	.944
		10	97.7					-0.4	.904	+0.1	.999	+0.1	>.999
	2	0	96.7					0	>.999	+0.8	.695	+0.1	>.999
		10	95.0					+0.5	.776	+0.3	.975	-0.8	.466
b.a5.1	2	0	92.6							0	>.999	+1.3	.253
		10	92.9							-0.8	.891	-1.5	.373
b.a6.2	2	0	95.2			+0.5	.992	-0.9	.895	-0.7	.963	-1.3	.630
		10	92.1			-0.4	.997	-1.8	.254	-0.3	>.999	-0.4	.999
	Treatment culture duration (days)	IL-1 α co-treatment (ng/ml)	Mean % cell viability of control	Difference (Δ) in mean % cell viability between control and treatment and significance level of the difference (P*)									
				Zol 5x10 ⁻⁷ M		Zol 10 ⁻⁶ M		Zol 10 ⁻⁵ M		Zol 10 ⁻⁴ M			
				Δ	P	Δ	P	Δ	P	Δ	P		
						-4.3	.002**			-7.4	<.001**		
						-2.4	.463			-10.1	<.001**		
b.a2	1	0	94.4			-0.7	.129	-0.5	.277				
		10	94.8			-0.2	.996	-0.4	.847				
b.a4.1	1	0	98.2	+0.7	.129	-0.5	.277						
		10	97.7	-0.2	.996	-0.4	.847						
	2	0	96.7	+0.4	.957	-1.1	.379						
		10	95.0	+0.5	.763	0	>.999						
b.a5.1	2	0	92.6			+0.2	.997	-0.3	.987	-2.6	.003**		
		10	92.9			-1.1	.680	-1.5	.384	-1.9	.176		
b.a6.2	2	0	95.2			-1.2	.715	-1.3	.571	-5.7	<.001**		
		10	92.1			+0.1	>.999	+0.3	>.999	-3.5	.003**		
Zol= zoledronate; * As multiple experiments were performed the alpha level to indicate a significant difference between means was set at P=0.01 as described in section 2.3.2; ** Statistically significant difference at the P<0.01 level													

A final experiment was carried out to clarify effects for zoledronate 10^{-4} M. In experiment b.a6.2, tissue was sourced from 18 month-old heifers and treatment cultures were performed in serum supplemented medium for two days (Table 3.2). Zoledronate 10^{-4} M decreased cell viability in both basal and IL-1 α co-treated cultures by 5.7% ($P<0.001$) and 3.5% ($P=0.003$) respectively (Fig. 3.6; Table 3.4). Again, no effects were seen with lower zoledronate concentrations. Table 3.4 summarises the observed effects on chondrocyte viability of the zoledronate concentrations tested across the four experiments.

3.5.2 Effects of zoledronate on chondrocyte proliferation and proteoglycan synthesis with and without IL-1 α co-treatment

Effects of IL-1 α on chondrocyte proliferation and proteoglycan synthesis

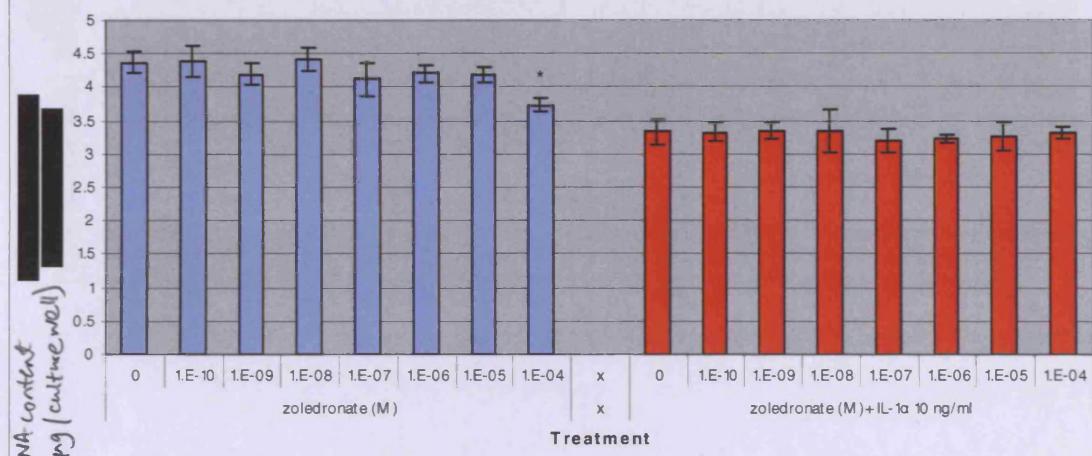
In controls, IL-1 α treatment reduced cell proliferation (measured by $^{3}\text{H-TdR}$ incorporation) by 66.1% compared to basal culture ($P<0.001$). Consistent with this finding, cell numbers were also lower with IL-1 α as indicated by a 14.7% decrease in bead DNA content ($P<0.001$). IL-1 α also reduced proteoglycan synthesis (measured by $^{35}\text{SO}_4$ incorporation) by 42.3% per culture well ($P<0.001$). When $^{35}\text{SO}_4$ incorporation was normalised for DNA content in the beads, the effect of IL-1 α was still evident (24.5% reduction; $P<0.001$) indicating an action on chondrocyte proteoglycan synthesis independent of an effect on cell numbers.

Effects of zoledronate on chondrocyte proliferation and proteoglycan synthesis without IL-1 α co-treatment

Zoledronate 10^{-4} M reduced cell proliferation by 8.1% ($P<0.001$) and bead DNA content by 14.7% ($P<0.001$) compared to control (Figs. 3.7 and 3.8). In addition, zoledronate 10^{-4} M inhibited proteoglycan synthesis by 29.5% per culture well ($P<0.001$; Fig. 3.9) and by 17.4% when values were normalised for DNA ($P<0.001$; Fig. 3.10). A 5% reduction in $^{35}\text{SO}_4$ incorporation per culture well was seen with 10^{-5} M zoledronate ($P=0.02$; Fig. 3.9) but this finding was not

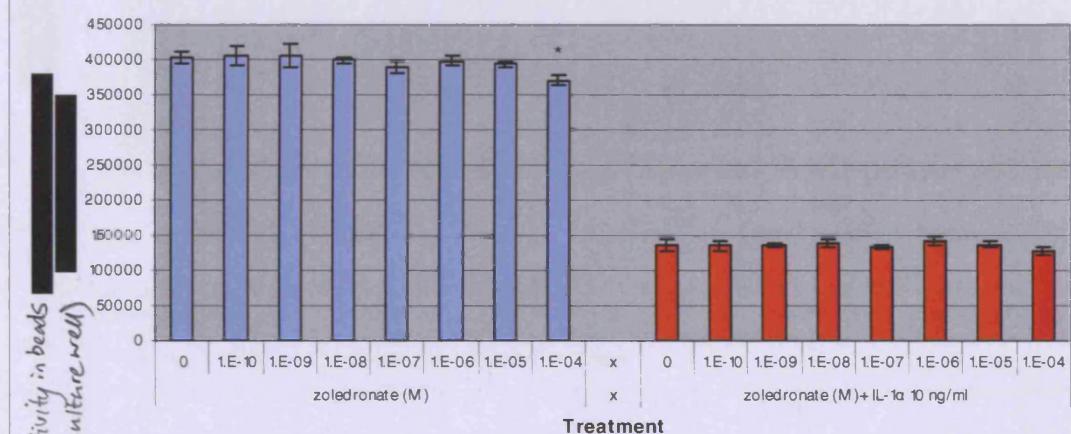
interpreted to be statistically significant because the data set did not distribute normally and the P value was not highly significant. No effects were seen with any of the other zoledronate concentrations tested compared to control.

Fig. 3.7. Exp. b.a6.1: Effects of zoledronate (1.E-10 M to 1.E-4 M) with and without IL-1 α co-treatment on the DNA content of chondrocytes cultured in alginate beads.



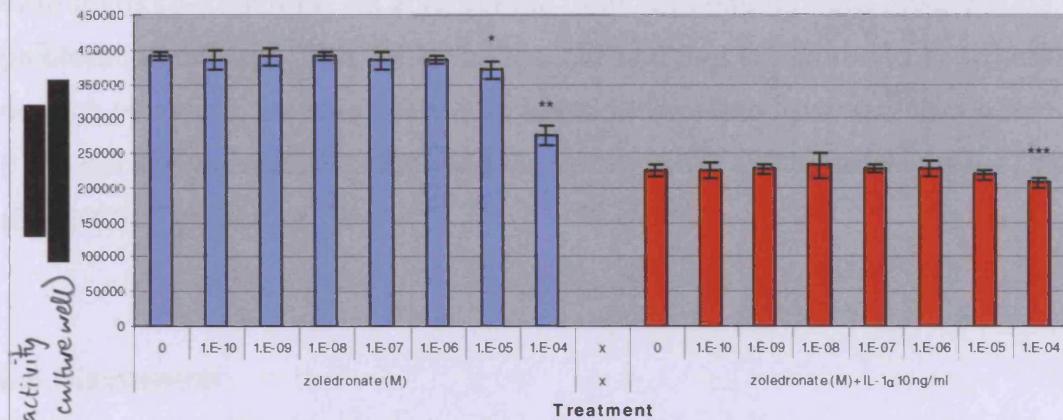
Bovine articular chondrocytes cultured in alginate beads in the presence of treatments for two days prior to analysis of DNA content. Values for bead DNA content per culture well shown as means \pm SD (n=6). *P<0.001 vs. zoledronate 0M.

Fig. 3.8. Exp. b.a6.1: Effects of zoledronate (1.E-10 M to 1.E-4 M) with and without IL-1 α co-treatment on the proliferation of chondrocytes cultured in alginate beads.



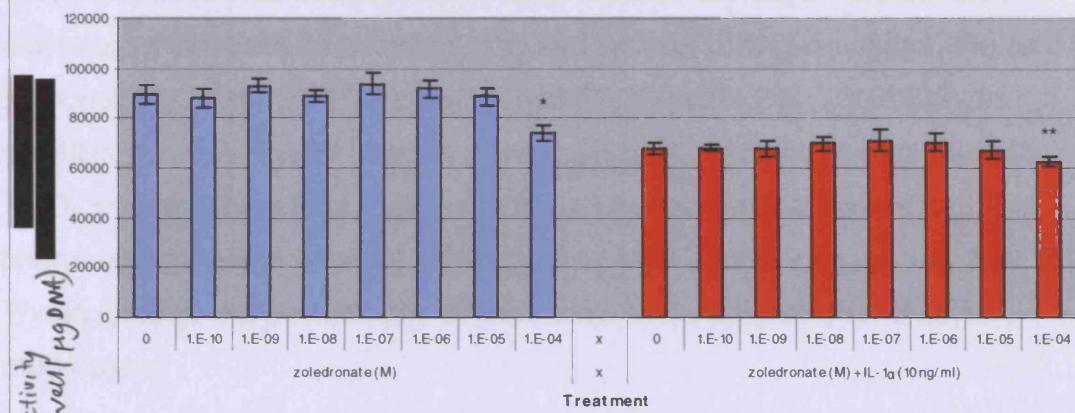
Bovine articular chondrocytes cultured in alginate beads in the presence of treatments and 3 H-TdR for two days. Chondrocyte proliferation assessed by amount of radioactive 3 H-TdR incorporated into DNA in beads at end of culture period. Values for radioactivity per culture well shown as means \pm SD (n=6). Cpm=counts per minute. *P<0.001 vs.

Fig. 3.9. Exp. b.a6.1: Effects of zoledronate (1.E-10 M to 1.E-4 M) with and without IL-1 α co-treatment on proteoglycan synthesis in chondrocytes cultured in alginate beads.



Bovine articular chondrocytes cultured in alginate beads in the presence of treatments and $^{35}\text{SO}_4$ for two days. Chondrocyte proteoglycan synthesis assessed by amount of radioactive $^{35}\text{SO}_4$ incorporated into proteoglycan in beads and medium at end of culture period. Values for radioactivity per culture well (beads + medium) shown as means \pm SD (n=6). cpm= counts per minute. *P=0.02 or **P<0.001 vs. zoledronate 0M. ***P=0.022 vs. zoledronate 0M + IL-1 α 10 ng/ml.

Fig. 3.10. Exp. b.a6.1: Effects of zoledronate (1.E-10 M to 1.E-4 M) with and without IL-1 α co-treatment on proteoglycan synthesis (normalised for DNA content) in chondrocytes cultured in alginate beads.



Bovine articular chondrocytes cultured in alginate beads in the presence of treatments and $^{35}\text{SO}_4$ for two days. Chondrocyte proteoglycan synthesis assessed by amount of radioactive $^{35}\text{SO}_4$ incorporated into proteoglycan in beads and medium at end of culture period. Values for radioactivity per culture well (beads + medium) normalised for DNA content shown as means \pm SD (n=6). cpm= counts per minute. *P<0.001 vs. zoledronate 0M. **P=0.025 vs. zoledronate 0M + IL-1 α 10 ng/ml.

Effects of zoledronate on chondrocyte proliferation and proteoglycan synthesis with IL-1 α co-treatment

With IL-1 α co-treatment, no zoledronate treatment effects were observed on cell proliferation or bead DNA content (Figs. 3.7 and 3.8) compared to IL-1 α -treated control. However, zoledronate 10 $^{-4}$ M did reduce proteoglycan synthesis by 8.0% per culture well ($P=0.022$; Fig. 3.9) or by 7.5% after normalising for DNA ($P=0.025$; Fig. 3.10).

3.6 Discussion

The aims behind the experiments described in this chapter were to determine whether zoledronate has adverse effects on chondrocytes and whether the bisphosphonate can stimulate chondrocyte proteoglycan synthesis. Treatment effects on chondrocyte viability, proliferation and proteoglycan synthesis were examined in bovine articular chondrocytes cultured in alginate beads with and without IL-1 α co-treatment as a stimulus for OA metabolic change.

Culture model

When chondrocytes isolated from bovine articular cartilage were cultured in alginate beads for up to two days with and without IL-1 α stimulation, the cells demonstrated good viability ranging from 92.1% to 98.2% (Table 3.3). In addition, the cells were shown to synthesise proteoglycan (as measured by $^{35}\text{SO}_4$ incorporation; Figs. 3.9 and 3.10), a phenotypic characteristic of chondrocytes, and proliferate (as measured by ^3H -TdR incorporation; Fig. 3.8). These findings support the use of this model to investigate chondrocyte metabolism.

For the two-day culture period, IL-1 α 10 ng/ml decreased cell viability in one out of three experiments (Table 3.3) and inhibited chondrocyte proliferation and proteoglycan synthesis in a single experiment (Figs. 3.7 to 3.10). These findings are consistent with previous reports of increased apoptosis, reduced cell proliferation and reduced proteoglycan synthesis following IL-1 α exposure in

bovine articular chondrocytes (Badger et al. 1999; Schuerwagh et al. 2003). Such adverse effects on chondrocytes support the concept that the IL-1 α plays a regulatory role in chondrocyte metabolism and is involved in promoting articular cartilage degradation during the OA process.

Effects of zoledronate on chondrocyte viability, proliferation and proteoglycan synthesis

The effects of zoledronate on chondrocyte viability (zoledronate 10 $^{-12}$ M to 10 $^{-4}$ M), and chondrocyte proliferation and proteoglycan synthesis (zoledronate 10 $^{-10}$ M to 10 $^{-4}$ M) have been evaluated under basal or IL-1 α co-treated conditions. Zoledronate 10 $^{-4}$ M consistently reduced cell viability under basal culture conditions (Figs. 3.1, 3.2, 3.5 and 3.6). With IL-1 α co-treatment, zoledronate 10 $^{-4}$ M decreased chondrocyte viability in three out of four experiments (Figs. 3.1, 3.2, 3.5 and 3.6). The absolute reductions in % cell viability were small in size ranging from 2.8% to 10.5%.

In one of the four experiments, and only under basal culture conditions, zoledronate 10 $^{-6}$ M treatment reduced chondrocyte viability by 4.6% (experiment b.a2; Fig. 3.1). Though this is an isolated finding, the significance level (P=0.002) indicates a genuine result. The lack of reproducibility may be due to an inter-experimental difference in the vulnerability of chondrocytes to toxicity arising from differences in methodology between experiments (see Table 3.2 for summary of methods). Adult bovine cartilage from the metacarpal articular surface of the MCP joint was used in all experiments, though other unknown characteristics of the tissue source may have been important for determining chondrocyte vulnerability *in vitro* (e.g. bovine breed, animal weight or history of joint injury). Alternatively, the lack of a pre-culture period to stabilise chondrocytes prior to commencing treatment in one of the four experiments (exp. b.a2 in which reduced chondrocyte viability was observed with zoledronate 10 $^{-6}$ M) could have been important. Chondrocytes that are cultured in alginate demonstrate initial cell loss (van Susante et al. 1995), suggesting

that chondrocytes that do not undergo initial pre-culture stabilisation may be more susceptible to toxic treatment effects.

Taken together the results from the four cell viability experiments indicate that zoledronate 10^{-4} M treatment is clearly adverse for the viability of bovine articular chondrocytes from the bovine MCP joint and this appears to be a biological effect across bovine animals of a similar age. These findings are consistent with reported effects of zoledronate on other cell types. At zoledronate concentrations 10^{-4} M and above, the bisphosphonate has been found to cause apoptosis of rabbit osteoclasts (Benford et al. 2001), human bone marrow stromal cells and human myeloma cells (Derenne et al. 1999). Furthermore clodronate, pamidronate and risedronate, all at the concentration of 10^{-3} M, have been shown to cause necrosis of bovine articular chondrocytes, (Van Offel et al. 2002). Thus, bisphosphonates as a class, at high concentrations, appear to reduce chondrocyte survival.

Zoledronate 10^{-4} M also inhibited chondrocyte proliferation under basal culture conditions (Figs. 3.7 and 3.8) and, with or without IL-1 α co-treatment, reduced proteoglycan synthesis (Figs. 3.9 and 3.10). The observed reductions in chondrocyte proliferation are consistent with previous reports which have found inhibitory effects of zoledronate on the proliferation of human foetal osteoblasts (zoledronate 10^{-5} M and 10^{-4} M) (Reinholz et al. 2000) and human myeloma cells (zoledronate 5×10^{-5} M to 10^{-4} M) (Derenne et al. 1999). Considering bisphosphonates as a class, the detrimental effects of zoledronate on chondrocyte proteoglycan synthesis are similar to reported inhibitory effects of etidronate and clodronate on canine cartilage proteoglycan synthesis (Palmoski and Brandt 1978) but discordant with a study that found a stimulatory effect of clodronate on rabbit chondrocyte proteoglycan synthesis (Guenther et al. 1979).

The mechanisms of action of the observed adverse effects of zoledronate on chondrocyte viability, proliferation and proteoglycan synthesis are unknown.

Reduced levels of chondrocyte viability may occur via stimulation of cell apoptosis. Such an effect might occur via inhibition of FPP synthase in the mevalonate pathway, which is the probable mechanism behind induction of rabbit osteoclast apoptosis seen with zoledronate treatment (Benford et al. 2001). Chelation of divalent cations by zoledronate is a possible mechanism for the inhibition of chondrocyte proteoglycan synthesis since calcium ions are stimulatory for proteoglycan synthesis in cartilage (Shulman and Opler 1974).

Overall, the results from the series of experiments described in this chapter indicate that zoledronate 10^{-4} M is detrimental for bovine articular chondrocytes from the MCP joint. This concentration was used to limit the upper range of subsequent experiments that evaluated the effects of the bisphosphonate on proteoglycan metabolism. The findings also have implications for the clinical use of zoledronate in that it would be advisable to avoid exposing cartilage to high concentrations of the bisphosphonate.

No information was found to support the hypothesis that zoledronate can enhance on cartilage proteoglycan metabolism during the OA process. Zoledronate treatments at concentrations 10^{-10} M through 10^{-4} M were not observed to stimulate chondrocyte proteoglycan synthesis, nor demonstrate any protective effects on the inhibitory actions of IL-1 α . The negative findings suggest that if zoledronate can improve the retention of cartilage proteoglycan in OA, the mechanism may be through inhibition of proteoglycan degradation.

3.7 Conclusions

- Culture of bovine articular chondrocytes in alginate beads provides a model for studying cell metabolism in which chondrocytes remain viable, proliferate and synthesise proteoglycan. Addition of IL-1 α to the culture system produces adverse effects on chondrocyte proliferation and proteoglycan synthesis and, possibly, chondrocyte viability.

- Zoledronate 10^{-4} M has detrimental effects on bovine articular chondrocyte viability, proliferation and proteoglycan synthesis. It would be advisable to avoid exposing cartilage to high concentrations of zoledronate during clinical use.
- Since adverse effects were observed, zoledronate 10^{-4} M was used as the upper limit of the concentration range to investigate in further in vitro studies of chondrocyte or cartilage metabolism. The lower end of this range, zoledronate 10^{-10} M, was defined by zoledronate concentrations reported to have effects on cell or enzyme processes in vitro (Table 3.1).
- No evidence was demonstrated to support the hypothesis that zoledronate can conserve cartilage GAG content during the OA process through stimulating chondrocyte proteoglycan synthesis or preventing the inhibitory effect of IL-1 α on proteoglycan synthesis. An alternative mechanism of action for zoledronate that has yet to be investigated is an inhibitory effect on proteoglycan degradation in cartilage.

Chapter 4. Effects of zoledronate on IL-1 α -stimulated proteoglycan degradation in bovine articular cartilage explants

This chapter describes experiments that tested for effects of zoledronate on IL-1 α -stimulated proteoglycan degradation in bovine cartilage explants.

4.1 Introduction

The investigations described in this chapter were performed to continue testing the hypothesis that zoledronate can modify cartilage metabolism to reduce the loss of cartilage GAG during the OA process. Results from investigations detailed in Chapter 3 do not indicate that zoledronate has a beneficial effect on the amount of proteoglycan synthesised by chondrocytes. In this chapter attention has turned towards examining for an effect on the other side of the metabolic balance: the degradation of proteoglycan in cartilage.

MMPs and aggrecanases are proteinases implicated in the degradation of aggrecan and type II collagen in OA cartilage (discussed in section 1.2.3.5) and there has been considerable interest in inhibiting the activities of these enzymes as a therapeutic strategy to prevent joint destruction in arthritic disorders including OA (Clark and Parker 2003; Elliott and Cawston 2001). At the biochemical level, studies have demonstrated that zoledronate and other bisphosphonates can inhibit the activities of various MMPs at bisphosphonate concentrations in the range 2×10^{-5} M to 10^{-3} M (Heikkila et al. 2002; Konttinen et al. 1999; Teronen et al. 1997a; Teronen et al. 1997b; Teronen et al. 1999) (Table 1.9). In addition, since MMPs and aggrecanases share common inhibitors (Hughes et al. 1998), it is possible that bisphosphonates can also inhibit aggrecanase activity.

At the cell or tissue level, bisphosphonates other than zoledronate have been found to have effects on chondrocyte-mediated proteinase activity, though findings are conflicting as to the direction of effect. Pamidronate was observed

to increase collagenase activity (Evequoz et al. 1985) and proteoglycan degradation (Couchman and Sheppeard 1986); clodronate to decrease collagenase activity (McGuire et al. 1982); tiludronate to reduce proteinase activity (Emonds-Alt et al. 1985); and etidronate to both increase collagenase activity (Evequoz et al. 1985) and decrease proteinase activity (Emonds-Alt et al. 1985; McGuire et al. 1982). The inhibitory effects on chondrocyte-mediated proteinase activity were observed at bisphosphonate concentrations in the range 10^{-5} M to 10^{-3} M (Table 1.9).

It is not known whether zoledronate can inhibit cartilage proteoglycan degradation at the tissue level. The principal investigations described in this chapter have sought this information in bovine articular cartilage explants stimulated with IL-1 α to induce GAG release (model described in section 2.2.1).

4.2 Experimental hypothesis

Zoledronate 10^{-10} M to 10^{-4} M reduces IL-1 α -stimulated GAG release from bovine articular cartilage explants

4.3 Experimental objectives

- i) Culture bovine articular cartilage explants with and without zoledronate (10^{-10} M to 10^{-4} M) and with and without 10 ng/ml IL-1 α
- ii) Measure GAG content in explant and in medium

4.4 Materials and methods

4.4.1 Tissue culture and treatments

Bovine cartilage explants prepared and pre-cultured as detailed in section 2.3.1.1. Single explants were placed in individual wells of 24-well tissue culture plates (Orange Scientific NV/SA, Braine-l'Alleud, Belgium). One ml of serum-free DMEM supplemented with or without 10 ng/ml recombinant human IL-1 α (tebu-bio, Peterborough, UK) and various concentrations of zoledronate (2-[imidazol-1-yl]-hydroxy-ethylidene-1,1-bisphosphonic acid, disodium salt, 4.75 hydrate; MW 401.6; donated by Novartis Pharma AG, Basel, Switzerland) was added to each well and explants were maintained in culture for four days. A four-day IL-1 α stimulation period was used based on previously described methods in studies of aggrecan catabolism (Caterson et al. 2000; Little et al. 1999). At the end of the culture period, the cartilage explants (approx wet weight 10-15 mg) and conditioned medium were collected and stored separately at -20°C until biochemical analysis of GAG content.

Five experiments were performed to test for effects of zoledronate. For each separate experiment, cartilage was sourced from the metacarpal surfaces of a single MCP joint (Fig. 4.1). Tissue source characteristics and the zoledronate concentrations evaluated for the five experiments are detailed in Table 4.1.

During the course of the experiments, notable intra-experimental variability in cartilage GAG release was encountered (described more fully below). In order to define this variability a preliminary experiment was performed to examine the effect of topographical sampling on IL-1 α -stimulated GAG release. The metacarpal articular surface was arbitrarily divided into three equal transverse bands (dorsal, central and palmar) and cartilage explants were sampled from these three regions (Fig. 4.1). Explants were cultured with and without IL-1 α 10 ng/ml as described above.

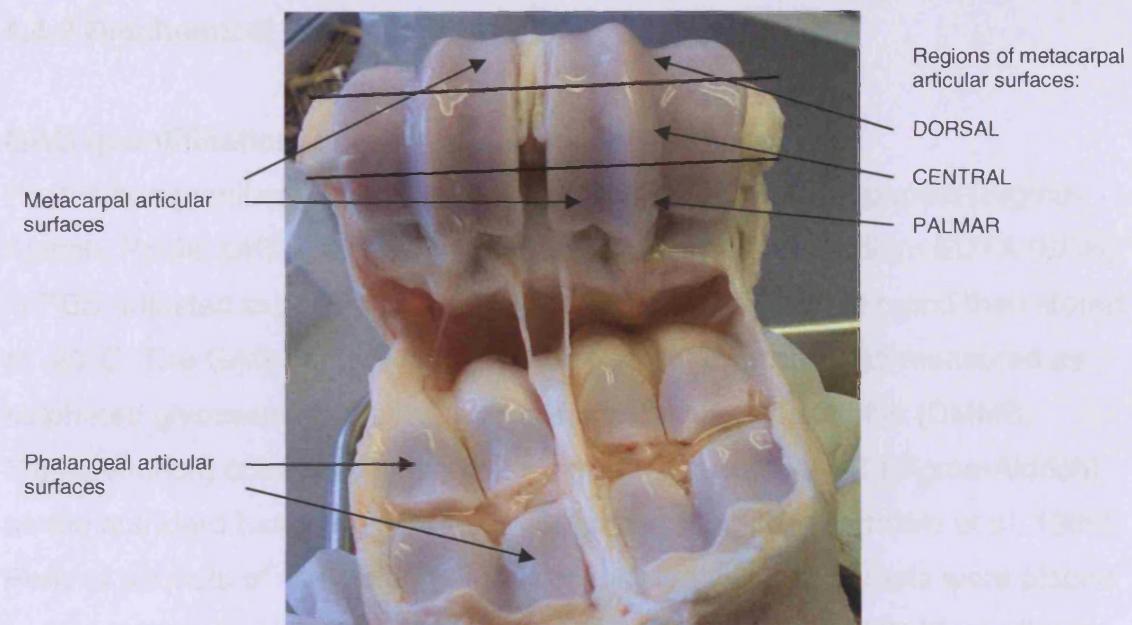


Fig. 4.1. Photograph showing the articular surfaces of a bovine metacarpophalangeal joint. Joint capsule and ligaments have been dissected to open joint and reveal articular surfaces. For experiments examining effects of zoledronate on cartilage, explant samples were taken only from the metacarpal surface. For an experiment investigating topographical differences in cartilage metabolism, the metacarpal articular surfaces of a single joint were arbitrarily divided into three equal transverse bands (dorsal, central and palmar) and separate pools of explant samples were taken from each of these regions.

Table 4.1. Summary of tissue source characteristics and zoledronate concentrations tested for experiments evaluating the effects of zoledronate on basal and IL-1 α -stimulated GAG release in bovine articular cartilage explants

Exp.	Animal tissue source		Number of replicates	Zoledronate concentration tested (M)						
	Age	Sex				10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
b.e1	18 months	N/A	n=2			10^{-8}	10^{-7}	10^{-6}	10^{-5}	10^{-4}
b.e2.b	16 months	heifer	n=6		10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	
b.e2.w	16 months	heifer	n=6		10^{-9}	10^{-8}	10^{-7}	10^{-6}	10^{-5}	
b.e3.2	20-24 months	steer	n=6	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}		
b.e3.3	20-24 months	steer	n=6	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}		

4.4.2 Biochemical analysis

GAG quantification

Post culture cartilage explants were digested with 125 µg/ml papain (Sigma-Aldrich, Poole, UK), 5 mM cysteine-HCl (BDH) and 5 mM sodium EDTA (BDH) in PBS adjusted to pH 6 with 1M NaOH (BDH) at 60°C for 12 h and then stored at -20°C. The GAG content of papain digests and medium was measured as sulphated glycosaminoglycan using the dimethylmethylen blue (DMMB; Sigma-Aldrich) colorimetric assay and chondroitin sulphate-C (Sigma-Aldrich) as the standard based on previously described methods (Farndale et al. 1986). Forty µl aliquots of standard in deionised distilled water or sample were placed in separate wells of 96 well flat bottom EIA microtitre plates (MP Biomedicals; Cambridge, Cambs, UK). A 250 µl aliquot of a solution containing 16 µg/ml DMMB in 1% (v/v) ethanol (BDH), 29.5 mM NaOH and 0.343% (v/v) formic acid (BDH) was added to each well and absorbance at 540 nm (A_{540}) measured using a microplate reader (Bio-Rad model 3550; Bio-Rad Laboratories, Hemel Hempstead, Herts, UK). Reproducibility: intra-assay CV 0.37%; inter-assay CV 1.96%. To exclude the possibility of an effect of zoledronate on this assay, A_{540} was measured on solutions containing 20 mcg/ml chondroitin sulphate-C and/or zoledronate 10^{-7} M to 10^{-4} M. Zoledronate did not demonstrate an effect on A_{540} . The amount of GAG release was expressed as a percentage and calculated as follows:

$$\text{\%GAG release} = \text{medium GAG content} / (\text{medium GAG content} + \text{explant GAG content})$$

4.4.3 Statistical analyses

Statistical analyses were performed as described in section 2.3.2. For data relating to experiments evaluating zoledronate treatment, multiple comparisons between zoledronate-treated and untreated control were made with Dunnett's t test. For the topographical sampling experiment multiple comparisons between sample regions were made using the Bonferroni test.

4.5 Results

Effects of IL-1 α on GAG release (Table 4.2)

Control bovine articular cartilage explants released small amounts of GAG in basal culture (mean GAG release ranged from 5.9% to 15.1%). For IL-1 α -stimulated controls, mean GAG release ranged from 36.9% to 62.9%.

Differences in GAG release between IL-1 α -treated controls and basal controls were significant at the P<0.001 level.

Table 4.2. GAG release from bovine articular cartilage explants with and without IL-1 α stimulation in controls.

Experiment	Mean %GAG release from controls (SD)		P value for difference between means
	Control	IL-1α-treated control	
b.e1	15.1 (1.1)*	36.9 (1.9)*	N/A ⁺
b.e2.b	11.5 (4.4)**	60.5 (15.2)**	P<0.001
b.e2.w	13.1 (3.9)**	62.9 (9.0)**	P<0.001
b.e3.2	5.9 (0.9)**	46.1 (11.7)**	P<0.001
b.e3.3	7.2 (0.9)**	59.8 (5.3)**	P<0.001

*n=2. **n=6. ⁺Statistical testing not performed since n=2.

Effects of zoledronate on GAG release with and without IL-1 α stimulation

The first experiment performed to evaluate effects of zoledronate was experiment b.e1. Though the experiment had been designed to be performed in triplicate (n=3), a technical issue meant that one set of samples was unavailable for GAG biochemical analysis. As only two observations were available for each treatment group (n=2) and the data also exhibited marked inhomogeneity of variance (Fig. 4.2), statistical comparisons were not performed.

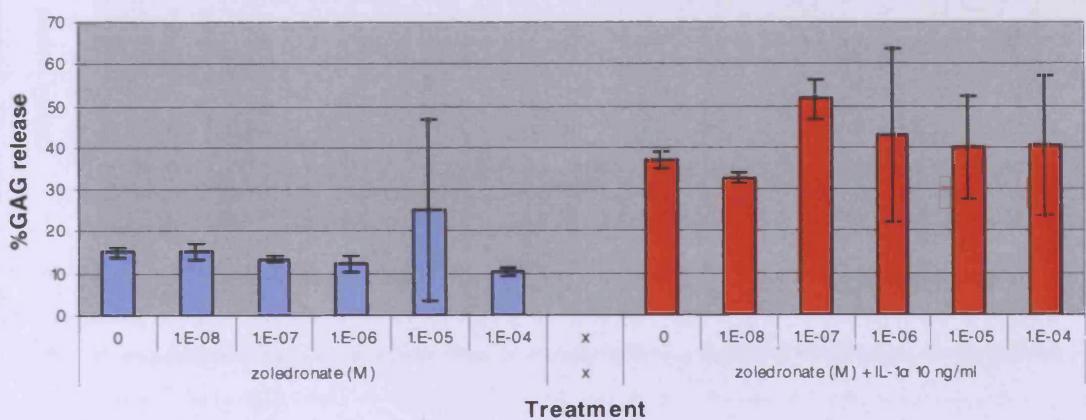
In the four other experiments (Figs. 4.3 to 4.6), the effects of zoledronate at concentrations 10^{-10} M through to 10^{-5} M were examined in the culture model. No effects of the bisphosphonate were observed on either unstimulated or IL-

1 α -stimulated GAG release. The intra-experimental co-efficient of variation in IL-1 α -stimulated GAG release, the main outcome measure of interest, was found to range from 8.9% to 25.4% in the four experiments (Table 4.3).

Table 4.3. Intra-experimental variation in IL-1 α -stimulated GAG release from bovine articular cartilage explants.

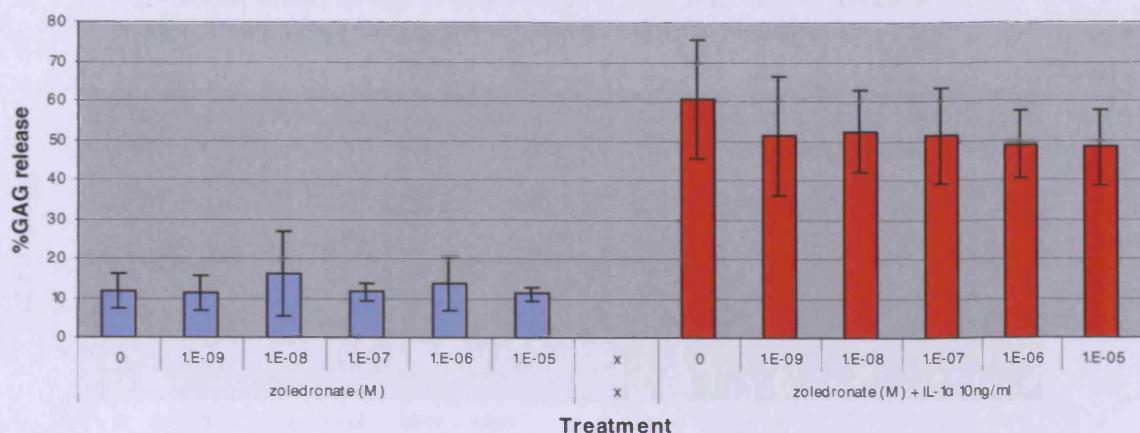
Experiment	Co-efficient of variation (CV) for IL-1 α -stimulated GAG release
b.e2.b	25.1%
b.e2.w	14.3%
b.e3.2	25.4%
b.e3.3	8.9%
Mean CV	18.4%

Fig. 4.2. Exp. b.e1: Effects of zoledronate (1.E-8 M to 1.E-4 M) on basal and IL-1 α -stimulated GAG release in bovine cartilage explants.



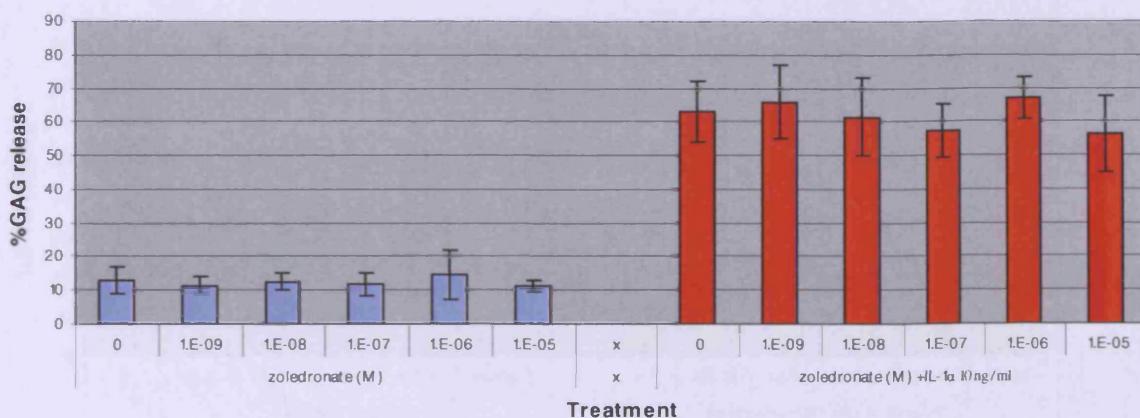
Bovine articular cartilage explants cultured in the presence of treatments for four days. Values for %GAG release shown as means \pm SD (n=2). Statistical comparisons of means not performed as n=2.

Fig. 4.3. Exp. b.e2.b: Effects of zoledronate (1.E-9 M to 1.E-5 M) on basal and IL-1 α -stimulated GAG release in bovine cartilage explants



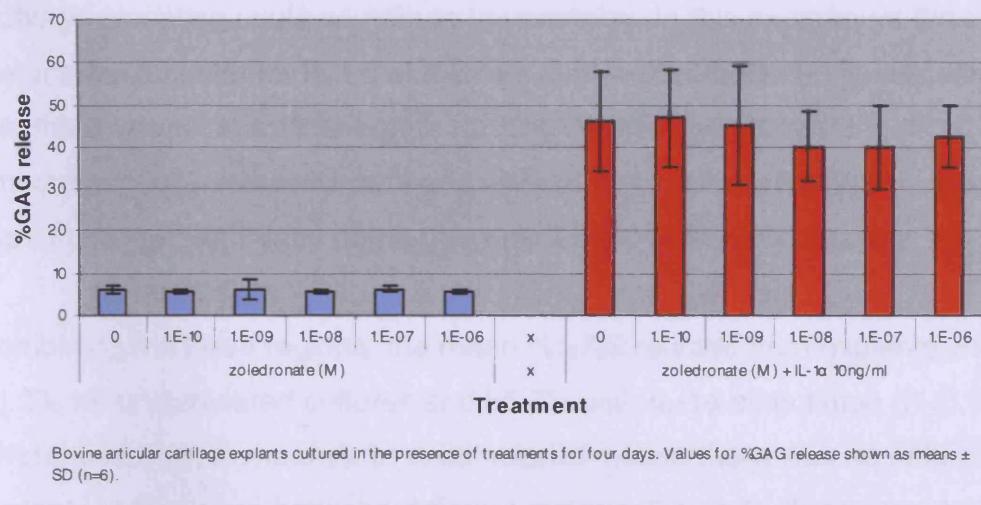
Bovine articular cartilage explants cultured in the presence of treatments for four days. Values for %GAG release shown as means \pm SD (n=6).

Fig. 4.4. Exp. b.e2.w: Effects of zoledronate (1.E-9 M to 1.E-5 M) on basal and IL-1 α -stimulated GAG release in bovine cartilage explants.



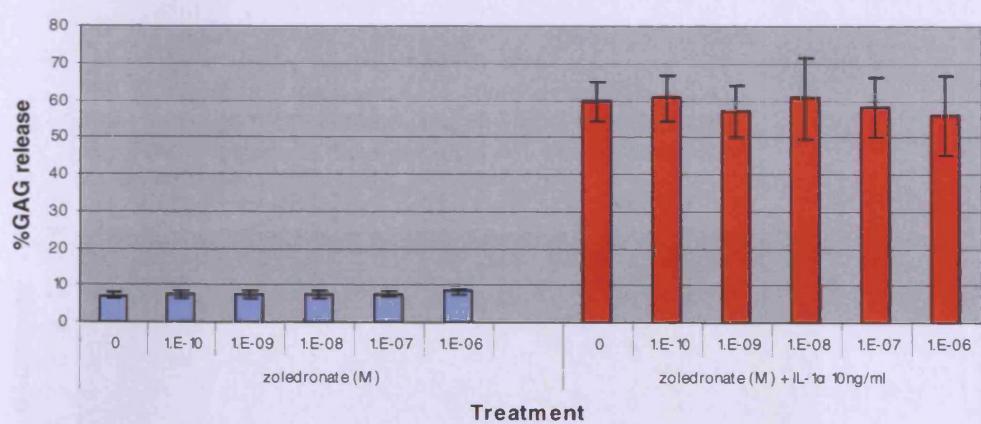
Bovine articular cartilage explants cultured in the presence of treatments for four days. Values for %GAG release shown as means \pm SD (n=6).

Fig. 4.5. Exp. b.e3.2: Effects of zoledronate (1.E-10 M to 1.E-6 M) on basal and IL-1 α -stimulated GAG release in bovine cartilage explants.



Bovine articular cartilage explants cultured in the presence of treatments for four days. Values for %GAG release shown as means \pm SD (n=6).

Fig. 4.6. Exp. b.e3.3: Effects of zoledronate (1.E-10 M to 1.E-6 M) on basal and IL-1 α -stimulated GAG release in bovine cartilage explants.



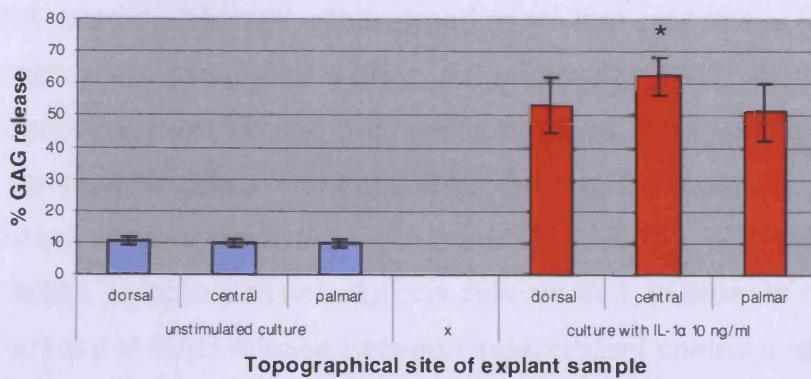
Bovine articular cartilage explants cultured in the presence of treatments for four days. Values for %GAG release shown as means \pm SD (n=6).

Variation in GAG release across the joint surface

The marked variation in GAG release of cartilage explants from a single articular surface prompted an experiment to examine whether the site of cartilage sampling could contribute to variability. In this experiment the rounded metacarpal articular surfaces of a single joint were arbitrarily visually divided into three equal transverse bands (dorsal, central and palmar; Fig. 4.1). The amounts of GAG released, with and without IL-1 α stimulation, from cartilage explants taken from each of the three joint regions were compared.

Combining the three regions, the mean %GAG release from explants was 10.2% for unstimulated cultures and 55.7% with IL-1 α stimulation ($P<0.001$ for difference between means). In unstimulated culture there was no difference in explant GAG release between different regions (Fig. 4.7). IL-1 α -stimulated GAG release from explants sampled from the central region (mean 62.5%) was greater than that for explants from the dorsal region (mean 53.3%; $P=0.026$) or palmar region (mean 51.3%; $P=0.005$). The stimulated GAG release for the latter two regions did not differ.

Fig. 4.7. Exp. b.e3.1: Basal and IL-1 α -stimulated GAG release from bovine cartilage explants sampled from dorsal, central and palmar regions of the metacarpal articular surfaces of a single MCP joint.



Bovine articular cartilage explants cultured for four days. Values for %GAG release shown as means \pm SD ($n=6$).
 $*P=0.026$ vs. dorsal with IL-1 α and $P=0.005$ vs. palmar with IL-1 α

4.6 Discussion

Effects of IL-1 α in the culture model

In the series of experiments, IL-1 α 10 ng/ml exposure consistently stimulated GAG release in cartilage explants compared to control cultures (Table 4.2). This catabolic response is consistent with previous reports (Caterson et al. 2000; Little et al. 1999) and supports the use of the culture model to investigate cartilage proteoglycan degradation.

Effects of zoledronate on cartilage GAG release with and without IL-1 α stimulation

Zoledronate treatment at concentrations 10^{-10} M to 10^{-5} M did not demonstrate any beneficial effects on the amount of GAG released from cartilage in the absence or presence of IL-1 α stimulation. Since separate experiments utilised tissue sourced from different animals (Table 4.1), the negative finding is more likely to be biologically relevant. Thus, in this series of experiments, no information was demonstrated to support the hypothesis that zoledronate can modify cartilage metabolism to reduce the loss of cartilage GAG during the OA process.

Intra-experimental variation for the culture model

However, these results need to be interpreted in light of the marked variability in GAG release observed within experiments that could have limited sensitivity for detecting treatment effects (Figs. 4.2 to 4.6; Table 4.2). Across the four experiments in which n=6 per treatment group, the intra-experimental coefficient of variation for IL-1 α -stimulated GAG release, the main outcome measure of interest, ranged from 8.9% to 25.4% (mean 18.4%). With these levels of variation, sample size calculations indicate that, in order to detect a 10% difference in GAG release between independent control and treatment groups ($\alpha=0.05$) with 80% power, sample sizes of at least 13 (n=13) or up to 102 (n=102) per treatment group would be needed. Clearly the experiments that

have been performed were underpowered to find zoledronate treatment effects that produced 10% differences in IL-1 α -stimulated GAG release.

One possible source for the observed variability was experimental technique. In initial experiments, relative inexperience with laboratory technique may have introduced between-sample variation. However, even towards the later stages of the project, intra-experimental variation remained notable (Figs. 4.5 and 4.6). Interestingly, the cutting of articular cartilage explants has been found to have metabolic effects such as the release basic fibroblastic growth factor (a proposed extracellular mechanotransducer involved in the regulation of tissue turnover) (Vincent et al. 2002; Vincent and Saklatvala 2006), the induction of IL-1 (Gruber et al. 2004) and also the stimulation of cell proliferation and matrix synthesis at the wound edge (Redman et al. 2004). Thus, differences in the way that cartilage explants were excised during tissue preparation may have contributed to between-sample variation. An alternative explanation is that, in a similar fashion to reported topographical differences in cartilage matrix composition across the joint surface (Bayliss et al. 1999; Bayliss et al. 2001; Brama et al. 2000), cartilage metabolism also exhibits topographical variation according to sample site.

Variation in IL-1 α -stimulated GAG release across the joint surface

In a single preliminary experiment, cartilage was sampled from the metacarpal articular surface of a single bovine MCP joint. Explants from the central region of the metacarpal articular surface released more GAG in response to IL-1 α 10 ng/ml compared to samples taken from either the dorsal or palmar region (Fig 4.7). This sampling effect on IL-1 α -stimulated GAG release offers a partial explanation for the high variability that was observed previously.

The results suggest that different regions of the bovine MCP joint have different vulnerabilities to IL-1 α -induced cartilage degradation. If these susceptibilities were to correlate with areas that commonly develop cartilage loss in OA, this would provide an explanation for the focal nature of cartilage loss that is often

seen during the OA process. In support of this concept, GAG release in response to IL-1 β has been found to vary in cartilage from different anatomical locations on human OA knee joints (Barakat et al. 2002). However, another study that investigated susceptibilities of equine cartilage to various cytokines (including IL-1) in relation to regions of the joint with known high or low vulnerabilities to degeneration in OA did not observe regional differences in response to catabolic cytokines that could explain the localisation of focal cartilage degeneration in OA (Little et al. 2005).

How to test the general hypothesis further

Increasing the sample size would be one way to evaluate zoledronate for small 10% sized treatment effects on GAG release. However, the metacarpal articular surfaces of a single MCP joint only yield approximately 80 cartilage explants which would be inadequate since, from sample size calculations (see above this section), up to 102 samples per treatment group would be needed. Ways to increase numbers include pooling samples from both metacarpal and phalangeal joint surfaces, pooling samples from other MCP joints (either from the same animal or another animal) or taking smaller and more numerous cartilage explants from the articular surface, though it is likely that variability will be increased with each of these techniques. Additional evaluation of these methods to determine feasibility and, in addition, reproducibility of GAG release would be required prior to use of the model to continue testing the general hypothesis.

An alternative approach is to use a culture model in which sources of metabolic variation are minimised. In the next chapter, experiments are described that further test the general hypothesis in the bovine chondrocyte/ alginate bead culture system, a model that demonstrates less inherent variability for IL-1 α -stimulated GAG release than the bovine cartilage explant culture model.

4.7 Conclusions

- The culture of bovine articular cartilage explants with IL-1 α 10 ng/ml to stimulate GAG release provides an in vitro culture model for studying cartilage proteoglycan degradation.
- There is noticeable variability in IL-1 α -stimulated GAG release from cartilage sampled from the metacarpal articular surfaces of the bovine MCP joint. This variability may be partly explained by regional differences in the susceptibility of cartilage to IL-1 α across the joint surface.
- Zoledronate treatment in the concentration range 10^{-10} M to 10^{-5} M was not observed to reduce IL-1 α -stimulated GAG release from bovine articular cartilage explants. Thus, no evidence was found to support the hypothesis that zoledronate can modify cartilage metabolism to reduce the loss of cartilage GAG during the OA process.
- The studies were under-powered to investigate for small-sized effects (e.g. 10%) with zoledronate treatment. An alternative culture model, with less inherent variability, should be more suitable for this purpose.

Chapter 5. Effects of zoledronate on IL-1 α -stimulated proteoglycan degradation in bovine articular chondrocytes cultured in alginate beads

This chapter describes experiments that tested for the effects of zoledronate on IL-1 α -stimulated proteoglycan degradation in a chondrocyte-matrix culture model.

5.1 Introduction

In the experiments detailed in Chapter 4, zoledronate treatment (10^{-10} M to 10^{-5} M) was not seen to alter IL-1 α stimulated GAG release in bovine articular cartilage explants. However, a small-sized effect may have been missed since the studies were underpowered to detect 10% differences between treatment and control. One approach to pursuing the possibility of small-sized treatment effects is to use a culture model with less variability. In the cartilage explant culture model, possible sources for between-sample variability in IL-1 α -stimulated GAG release include (i) topographical variation in the susceptibility of cartilage across an articular surface to the effects of IL-1 α and (ii) variation introduced during tissue processing when cartilage explants are cut (discussed in section 4.6).

The bovine chondrocyte/ alginate bead model (described in section 2.3.1.2) offers the potential to remove or average-out these sources of variation. Since this culture technique involves isolating chondrocytes from cartilage, the existing matrix with its attendant variability is removed from the culture system. Subsequent mixing of the cell suspension redistributes and averages out metabolic cellular differences. For the study of chondrocyte-matrix interactions, the chondrocytes can then be cultured in the alginate beads to grow a cartilage-like matrix (Hauselmann et al. 1996b; Petit et al. 1996). Variation in the matrix newly synthesised by chondrocytes is likely to be minimal since all the cells come from the same homogenised pool.

The studies described in this chapter investigate the effects of zoledronate on IL-1 α -stimulated proteoglycan degradation in the bovine chondrocyte/ alginate bead culture model. Initially, some experiments are described that were performed to validate the model by characterising how stimulus duration and IL-1 α concentration affect GAG release.

5.2 Experimental hypotheses

A. To validate the culture model

- A1. The amount of GAG release from alginate beads containing a matrix established by bovine articular chondrocytes is dependent on the duration of stimulus with IL-1 α 10 ng/ml.**
- A2. The amount of GAG release from alginate beads containing a matrix established by bovine articular chondrocytes is dependent on the concentration of IL-1 α used as a stimulus.**

B. To evaluate zoledronate treatment effects

Zoledronate 10^{-8} M to 10^{-4} M reduces IL-1 α -stimulated GAG release from alginate beads containing a matrix established by bovine articular chondrocytes

5.3 Experimental objectives

- i) Culture bovine articular chondrocytes in alginate beads to establish an extracellular matrix.
- ii) Measure GAG release from alginate beads stimulated by IL-1 α 10 ng/ml as a function of time (one, two or five days). Select duration of IL-1 α

stimulus to use in further experiments.

- iii) Measure IL-1 α -stimulated GAG release from alginate beads as a function of IL-1 α concentration (0.05 ng/ml to 20 ng/ml). Select concentrations of IL-1 α to use for further experiments.
- iv) Measure IL-1 α -stimulated GAG release with and without zoledronate treatment at concentrations 10 $^{-8}$ M to 10 $^{-4}$ M in the culture model.

Note: Objective (iii) was undertaken late on in the studies. Prior to information on GAG release as a function of IL-1 α concentration being available for the model, IL-1 α 10 ng/ml was used as the stimulus for GAG release in experiments of objective (iv) based on the concentration of IL-1 α that was found to effectively stimulate GAG release in the cartilage explant model (section 4.5).

5.4 Materials and methods

5.4.1 Chondrocyte culture in alginate beads

Alginate beads containing isolated bovine chondrocytes were formed as detailed in Chapter 2, Materials and methods. **Culture to establish extracellular matrix:** Beads were cultured in 225 cm 2 tissue culture flasks maintained in DMEM + 10% or 20% FCS for 21 to 42 days with medium changes three times a week. Tissue source characteristics for chondrocytes and differences in culture conditions between experiments are summarised in Tables 5.2 and 5.3. **Treatment culture:** After the matrix-establishing culture period beads were washed 3 times for 10 minutes each in serum-free DMEM. Six beads were placed in individual wells of 24-well tissue culture plates to which was added 1 ml of serum-free DMEM supplemented with or without IL-1 α and with or without zoledronate treatments. Concentrations of IL-1 α and zoledronate used in different experiments are detailed in Tables 5.2 and 5.3. Each well represented one replicate and treatment cultures were performed replicates of three to six. Beads were maintained in medium for 1, 2 or 5 days. At the end of the treatment culture period beads and medium were harvested

and stored separately at -20°C until biochemical analysis. A typical time line for chondrocyte/ bead culture is shown in Table 5.1.

Table 5.1. Typical event time line for the bovine chondrocyte/ alginate bead culture model used in experiments detailed in Chapter 5.					
	Day of culture				
	-1	0	0-35	35-37	37
Event	Cartilage excised off joint surface and digested to release chondrocytes	Formation of alginate beads containing chondrocytes	Culture of beads in DMEM + 20% FCS with medium change 3x a week to establish matrix	Treatment culture of beads in serum-free DMEM \pm IL- α \pm Zol	Beads and medium harvested for analysis of GAG content
DMEM= Dulbecco's modified Eagle's medium; FCS= foetal calf serum; Zol= zoledronate; GAG= glycosaminoglycan					

5.4.2 Biochemical analysis

GAG quantification

Alginate beads were disrupted and digested in 1ml of 55 mM sodium citrate (BDH), 150 mM sodium chloride (Sigma), 5 mM cysteine hydrochloride, 5 mM EDTA (BDH) and 0.56 units/ml papain (Sigma), incubated at 60°C for 24 h (Enobakhare et al. 1996). Bead digest and medium GAG content was quantified using the DMMB dye method as described in chapter 4, Materials and methods, but with the dye adjusted to pH 1.5 to minimise alginate-dye complex formation according to previously reported adaptations (Enobakhare et al. 1996).

Reproducibility: mean intra-assay CV 0.38%; mean inter-assay CV 4.01%.

Measured GAG release was expressed as percentage released according to the following formula:

$$\% \text{GAG release} = \text{medium GAG content} / (\text{medium GAG content} + \text{bead GAG content})$$

Table 5.2. Summary of cell source characteristics and culture conditions for experiments evaluating IL-1 α -stimulated GAG release as a function of IL-1 α concentration or stimulus duration in the bovine chondrocyte/ alginate bead culture model.

Exp.	Function evaluated	Characteristics of cell source	Matrix-establishing culture period; %FCS used	Treatment culture conditions		
				Replicates	IL-1 α stimulus (ng/ml)	IL-1 α stimulus duration
b.a3.1	Duration of IL-1 α stimulus	1x MCP joint from a single heifer aged 20 months	21 days; 10% FCS	n=3	0, 10	1, 2 or 5 days
b.a6.5	Concentration of IL-1 α stimulus	7x MCP joints from 4 heifers aged 18 months	42 days; 20% FCS	n=4	0, 2, 10 or 20	2 days
b.a7.3	Concentration of IL-1 α stimulus	5x MCP joints from 5 heifers; ages unknown	23 days; 20% FCS	n=4	0, 0.5, 1, 2, 10 or 20	2 days
b.a7.4	Concentration of IL-1 α stimulus	5x MCP joints from 5 heifers*; ages unknown	25 days; 20% FCS	n=4	0, 0.05, 0.2, 0.5, 2 or 10	2 days

Exp.= experiment; MCP= metacarpophalangeal; FCS= foetal calf serum; *different joints to those used in Exp. b.a7.3

Table 5.3. Summary of cell source characteristics and culture conditions for experiments evaluating the effects of zoledronate treatment on IL-1 α -stimulated GAG release in the bovine chondrocyte/ alginate bead culture model.

Exp.	Characteristics of cell source	Matrix-establishing culture period; %FCS used	Treatment culture (all treatment cultures performed over 2 days)						
			Replicates	IL-1 α (ng/ml)	Zoledronate treatment concentrations tested (M)				
b.a3.2	1x MCP joint from a single heifer aged 20 months	28 days; 10% FCS	n=6	0, 10	10^{-8}	10^{-7}	5×10^{-7}		
b.a4.2	3x MCP joints from 2 bulls aged 13 months	22 days; 20% FCS	n=6	0, 10	10^{-8}	10^{-7}		10^{-6}	
b.a4.4	3x MCP joints from 2 bulls aged 13 months	29 days; 20% FCS	n=6	0, 10	10^{-8}	10^{-7}		10^{-6}	
b.a5.3	2x MCP joints from 2 heifers aged 24 and 29 months	31 days; 20% FCS	n=5	0, 10		10^{-7}		10^{-6}	10^{-5}
b.a6.3	7x MCP joints from 4 heifers aged 18 months	35 days; 20% FCS	n=6	0, 10		10^{-7}		10^{-6}	10^{-5}
b.a7.6	5x MCP joints from 5 heifers*; ages unknown	32 days; 20% FCS	n=3 or n=6	0, 0.05		10^{-7}		10^{-6}	10^{-5}

Exp.= experiment; FCS= foetal calf serum; MCP= metacarpophalangeal

5.4.3 Statistical analyses

Statistical analyses were performed as described in section 2.3.2. Comparisons of means between multiple groups were made using the Bonferroni test, except where comparisons were only between treatment groups and control in which case Dunnett's test was used. As previously detailed (section 2.3.2), the alpha level was set at $P=0.05$ for an hypothesis that was tested once and at $P=0.01$ for an hypotheses that was tested in multiple experiments.

A linear regression model was used to describe correlation between GAG release and IL-1 α concentration in experiments b.a7.3 (assumptions met for data normality, equality of variance and linearity).

5.5 Results

5.5.1 Culture model characteristics

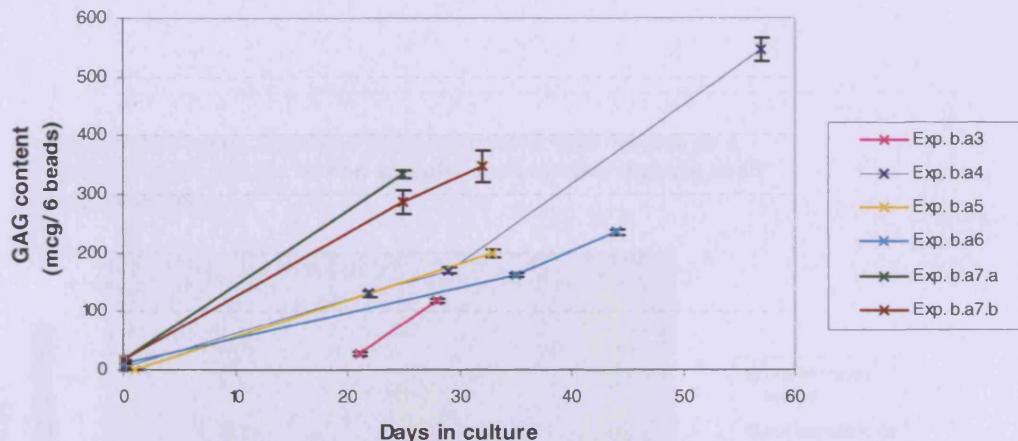
Culture of bovine articular chondrocytes in alginate beads to establish extracellular matrix (Fig. 5.1)

Culture of chondrocytes in alginate beads increased bead GAG content as a function of time, though the rate of GAG accumulation appeared to differ between experiments. After approximately one month of culture, GAG content ranged from 118 μ g to 347 μ g GAG per six beads.

GAG release as a function of IL-1 α stimulus duration (Fig. 5.2)

Experiment b.a3.1 investigated basal and IL-1 α -stimulated GAG release at various time points (one, two and five days). GAG was spontaneously released from the beads in basal culture, with similar amounts released for one-day and two-day culture (mean GAG release 29.1% and 26.3% respectively; $P=0.609$) but higher amounts at five days (mean GAG release 60.5%; $P<0.001$ vs. one-day or two-day culture).

Fig. 5.1. Matrix synthesis by bovine articular chondrocytes cultured in alginate beads: GAG content as a function of time. Results for six different experiments.



Bovine articular chondrocytes cultured in alginate beads in serum supplemented medium (10% foetal calf serum used in exp. b.a3 and 20% foetal calf serum used in all other exps.) Different animals were used to source chondrocytes between experiments. Culture period prior to assessment of bead GAG content varied between experiments and ranged from 21 days to 57 days. Values for bead GAG content shown as means \pm SD (n=6). Lines connecting data points are for illustrative purposes.

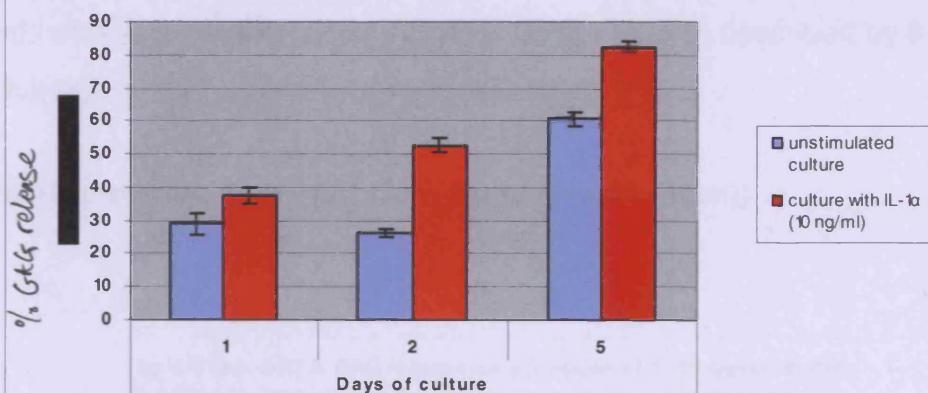
With IL-1 α treatment, the means for %GAG release after one-day, two-day or five-day culture were 37.4%, 52.6% and 82.5% respectively. At each time point IL-1 α stimulated GAG release compared to basal culture (significance levels for time points: one-day, P=0.027; two-day P<0.001; and five day P<0.001). IL-1 α -stimulated GAG release increased as a function of stimulus duration (two days vs. one day: P<0.001; five days vs. two days: P<0.001). Since the largest difference in GAG release between basal and IL-1 α stimulated culture was observed at the 2-day time point, this stimulation period was selected for use in subsequent experiments.

GAG release as a function of IL-1 α concentration (Figs. 5.3, 5.4, 5.5 and 5.6)

In the first of three experiments, mean GAG release with basal culture was 19.9%. Compared to basal culture, IL-1 α 2 ng/ml, 10 ng/ml and 20 ng/ml stimulated GAG release (mean values for GAG release 72.9%, 67.6% and 67.8% respectively, P<0.001 for all comparisons; Fig. 5.3). There was no difference in GAG release between IL-1 α 10 ng/ml and 20 ng/ml (P>0.999) but,

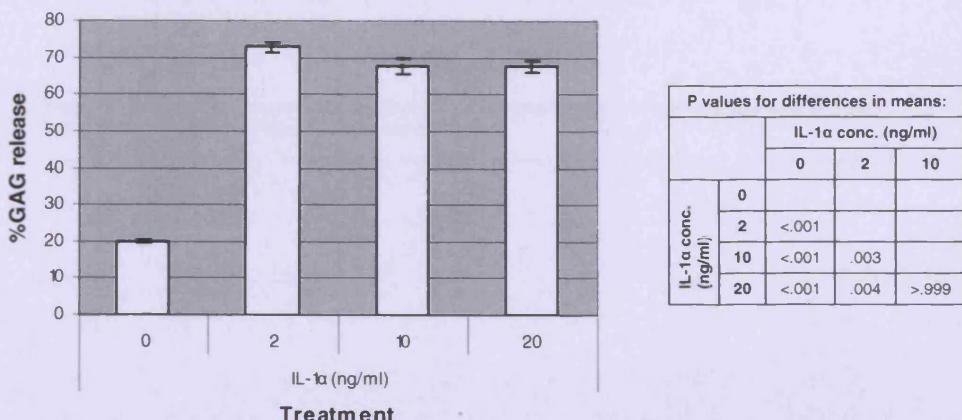
interestingly, both of these IL-1 α concentrations were less stimulatory than IL-1 α 2 ng/ml (P=0.003 and P=0.002 respectively).

Fig. 5.2. Exp. b.a3.1. Basal and IL-1 α -stimulated GAG release as a function of time in the bovine articular chondrocyte/ alginate bead culture model.



Alginate beads containing bovine articular chondrocytes and an established matrix cultured with and without IL-1 α stimulation for 1 day, 2 days or 5 days. Values for %GAG release from beads shown as means \pm SD (n=3). Mean %GAG release higher with IL-1 α stimulation than without at all time points (day 1, P<0.022; day 2, P<0.001; day 5, P<0.001). Mean unstimulated %GAG release higher at day 5 vs. day 1 (P<0.001) and day 5 vs. day 2 (P<0.001). Mean IL-1 α -stimulated %GAG release increases as a function of time (day 2 vs. day 1, P<0.001; day 5 vs. day 2, P<0.001)

Fig. 5.3. Exp. b.a6.5. GAG release as a function of IL-1 α concentration (2 ng/ml to 20 ng/ml) in the bovine articular chondrocyte/ alginate bead culture model.

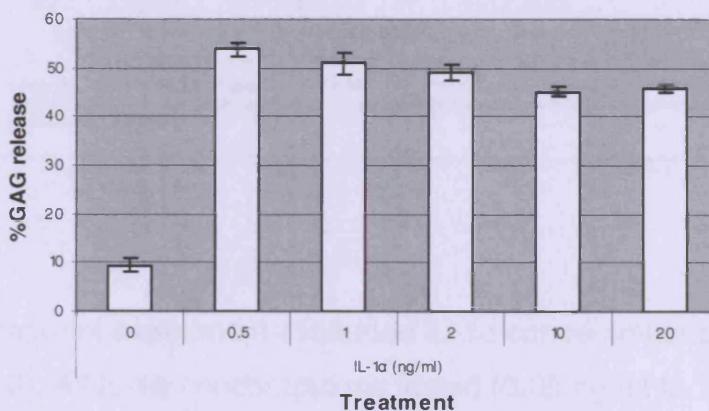


Alginate beads containing bovine articular chondrocytes and an established matrix cultured with treatments for 2 days. Values for %GAG release from beads shown as means \pm SD (n=4) on chart above left. P values for differences in mean %GAG release between treatments shown in table above right.

This unexpected finding led to a further experiment examining IL-1 α concentrations down to 0.5 ng/ml (Fig. 5.4). Again each of the IL-1 α concentrations tested caused an increase in GAG release compared to control ($P<0.001$ for all comparisons). Furthermore, in agreement with the previous experiment (b.a6.5), there appeared to be an inverse relationship between IL-1 α concentration and GAG release. For IL-1 α 0.5 ng/ml to 20 ng/ml, a simple linear regression analysis of $\log_{10}(\text{IL-1}\alpha \text{ concentration})$ and %GAG release indicated a significant correlation ($P<0.001$; $R^2= 0.79$; Fig. 5.5) described by the following equation:

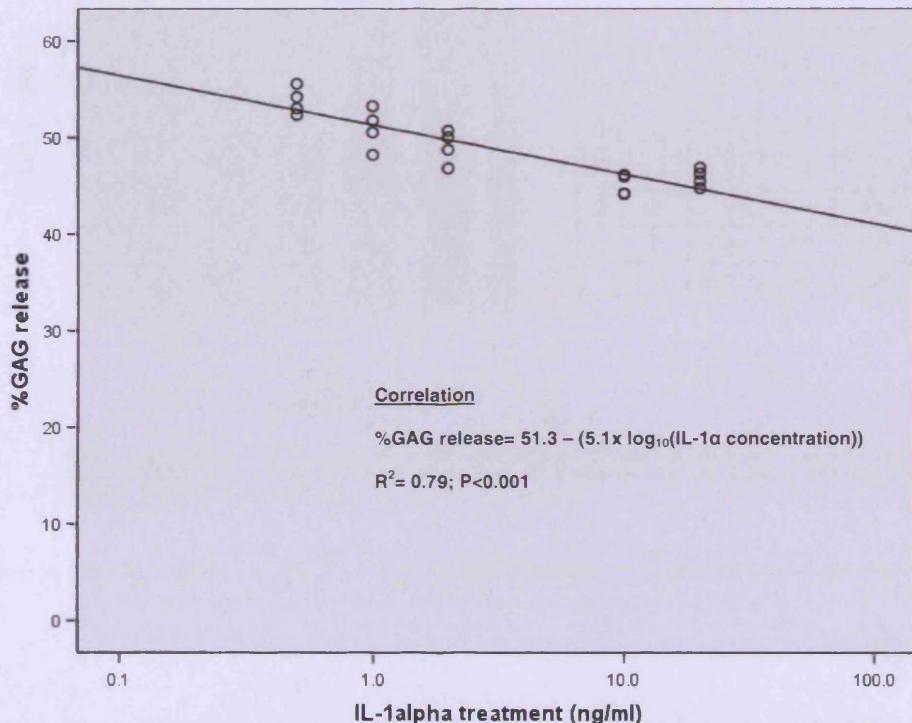
$$\text{\%GAG release} = 51.3 - (5.1 \times \log_{10}(\text{IL-1}\alpha \text{ concentration}))$$

Fig. 5.4. Exp. b.a7.3. GAG release as a function of IL-1 α concentration (0.5 ng/ml to 20 ng/ml) in the bovine articular chondrocyte/ alginate bead culture model.



Alginate beads containing bovine articular chondrocytes and an established matrix cultured with treatments for 2 days.
Values for %GAG release from beads shown as means \pm SD ($n=4$).

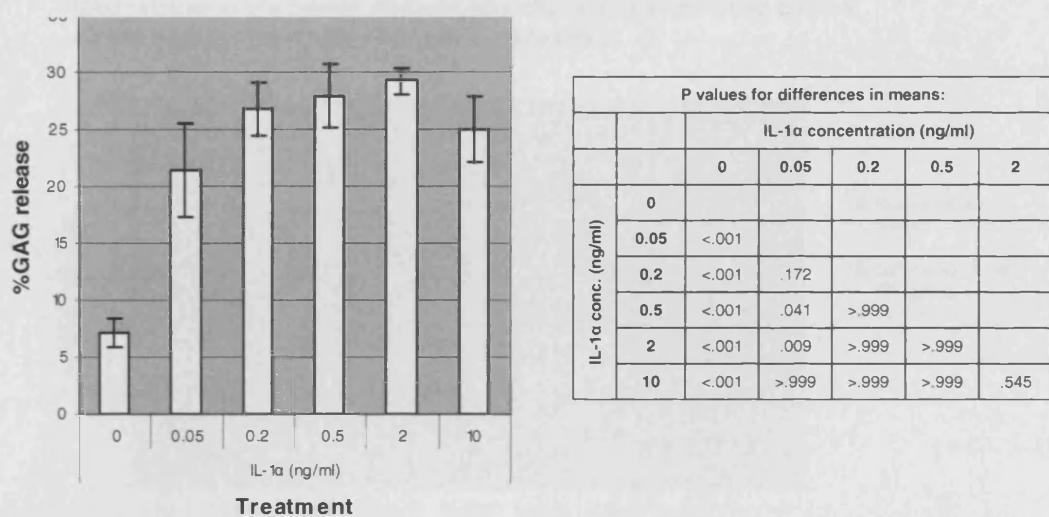
Fig. 5.5. Exp. b.a7.3. Linear regression model of GAG release as a function of \log_{10} IL-1 α concentration (0.5 ng/ml to 20 ng/ml) in the bovine articular chondrocyte/alginate bead culture model.



Linear regression modelling of data from exp. b.a7.3. Beads containing bovine articular chondrocytes and an established matrix cultured with treatments for 2 days. Data points represent %GAG release from beads treated with various concentrations of IL-1 α .

A subsequent experiment evaluated IL-1 α concentrations down to 0.05 ng/ml (Fig. 5.6). All IL-1 α concentrations tested (0.05 ng/ml to 10 ng/ml) compared to control stimulated GAG release ($P < 0.001$ for all comparisons). Graphically, there appeared to be a positive relationship between GAG release and IL-1 α concentration at the IL-1 α concentration range 0.05 ng/ml to 2 ng/ml (Fig.5.6). Less GAG release was stimulated by IL-1 α 0.05 ng/ml than by IL-1 α 0.5 ng/ml ($P=0.041$) or IL-1 α 2.0 ng/ml ($P=0.009$), though other comparisons between IL-1 α concentrations did not reveal any significant differences. Linear regression modelling was not performed since the data did not fulfil the linearity assumption (with or without log transformation).

Fig. 5.6. Exp. b.a7.4. GAG release as a function of IL-1 α concentration (0.05 ng/ml to 10 ng/ml) in the bovine articular chondrocyte/ alginate bead culture model.

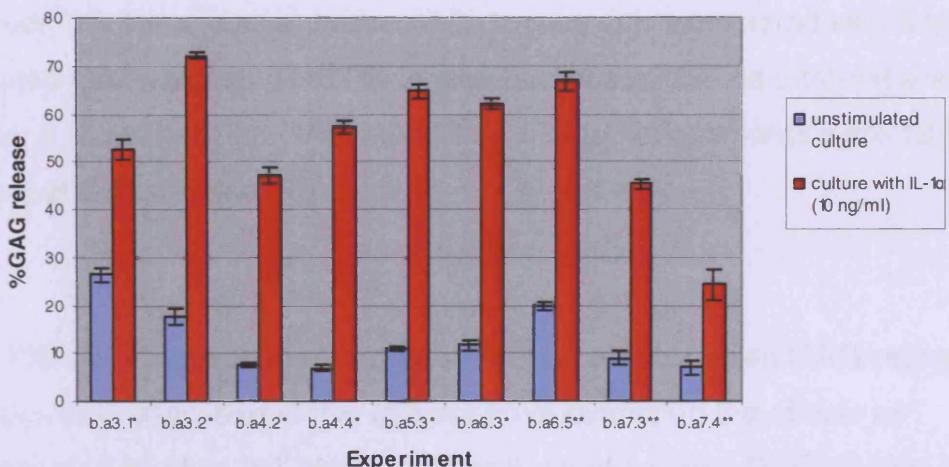


Beads containing bovine articular chondrocytes and an established matrix cultured with treatments for two days. Values for %GAG release from beads shown as means \pm SD ($n=4$) on chart above left. P values for differences in mean %GAG release between treatments shown in table above right.

Intra-experimental and inter-experimental variation in GAG release

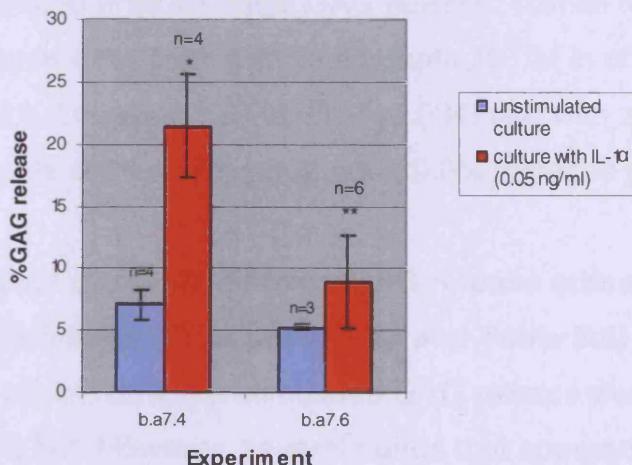
IL-1 α 10 ng/ml consistently stimulated GAG release from beads compared to basal culture across nine experiments ($P<0.001$ for comparisons in each experiment; Fig. 5.7). Intra-experimental CVs for unstimulated and IL-1 α -stimulated GAG release ranged from 3.8% to 21.5% (mean 9.1%) and 0.7% to 13% (mean 3.6%) respectively. The inter-experiment CVs for unstimulated and IL-1 α -stimulated GAG release were 52.6% and 26.5% respectively. Between-experiment differences for unstimulated or IL-1 α -stimulated GAG release were found to be highly significant ($P<0.001$ for both measures). Possible explanations for this striking inter-experimental variation are discussed below.

Fig. 5.7. Inter-experimental variation in basal and IL-1 α -stimulated (10 ng/ml) GAG release in the bovine articular chondrocyte/alginate bead culture model. Results from nine separate experiments.



Beads containing bovine articular chondrocytes and an established matrix cultured with and without IL-1 α 10 ng/ml for 2 days. Values for %GAG release from beads shown as means \pm SD (*n=6; **n=4).

Fig. 5.8. Basal and IL-1 α -stimulated (0.05 ng/ml) GAG release in the bovine articular chondrocyte/alginate bead culture model. Results from two separate experiments.



Beads containing bovine articular chondrocytes and an established matrix cultured with and without IL-1 α 0.05 ng/ml for 2 days. Values for %GAG release from beads shown as means \pm SD (numbers indicated on chart). *P<0.001 vs. unstimulated culture in exp. b.a7.4. **P=0.06 vs. unstimulated culture in exp. b.a7.6.

In two experiments (b.a7.4 and b.a7.6), IL-1 α 0.05 mg/ml was used to stimulate GAG release (Fig. 5.8). In experiment b.a7.4, IL-1 α 0.05 ng/ml significantly increased %GAG release compared to unstimulated culture ($P<0.001$). However, the same concentration of IL-1 α was only associated with a trend for increased GAG release ($P=0.06$) in experiment b.a7.6. The intra-experimental CVs for IL-1 α -stimulated GAG release for the two experiments were 19.2% (b.a7.4) and 42.2% (b.a7.6).

5.5.2 Effects of zoledronate on basal or IL-1 α -stimulated GAG release

Investigations described in this chapter have examined the effects of zoledronate 10^{-8} M to 10^{-4} M on basal or IL-1 α -stimulated GAG release in the bovine articular chondrocyte/ alginate bead culture model. Most experiments used IL-1 α at the concentration 10 ng/ml to stimulate GAG release. IL-1 α 0.05 ng/ml was employed as the stimulus in a single later experiment.

Effects of zoledronate on basal GAG release- six experiments (Figs. 5.9 to 5.14 and Table 5.4)

Compared to control, treatment with zoledronate 10^{-8} M to 10^{-4} M was not observed to effect basal GAG release. Trends for increases in basal GAG release were seen with zoledronate 10^{-7} M in one experiment out of six (b.a4.4; 10.6% increase vs. control; $P=0.034$) and with zoledronate 10^{-4} M in one experiment out of three (b.a6.3; 9.6% increase vs. control; $P=0.039$).

Effects of zoledronate on GAG release stimulated by IL-1 α 10 ng/ml- five experiments (Figs. 5.9 to 5.13 and Table 5.5)

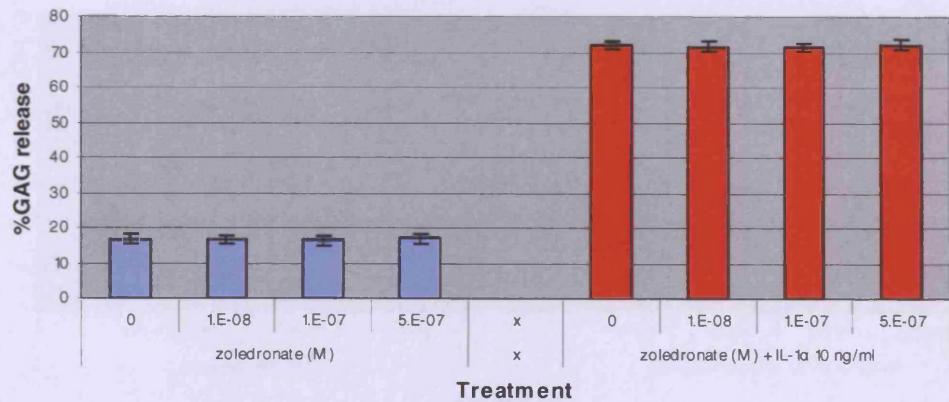
No effects on IL-1 α -stimulated GAG release were seen with zoledronate 10^{-8} M to 10^{-4} M. However, several trends that approached significance were apparent. In separate experiments increases in stimulated GAG release were found with zoledronate 10^{-6} M in one experiment of four (b.a4.4; 6.2% increase vs. control; $P=0.011$) and zoledronate 10^{-5} M in one of two experiments (b.a6.3; 4.8% increase vs. control; $P=0.012$). Decreases in stimulated GAG release were seen

with zoledronate 10^{-5} M (4% decrease vs. control; $P=0.033$) and zoledronate 10^{-4} M (4.3% decrease vs. control; $P=0.022$) in one of two experiments (b.a5.3; both findings in the same experiment).

Effects of zoledronate on GAG release with IL-1 α 0.05 ng/ml co-treatment-single experiment (Fig. 5.14)

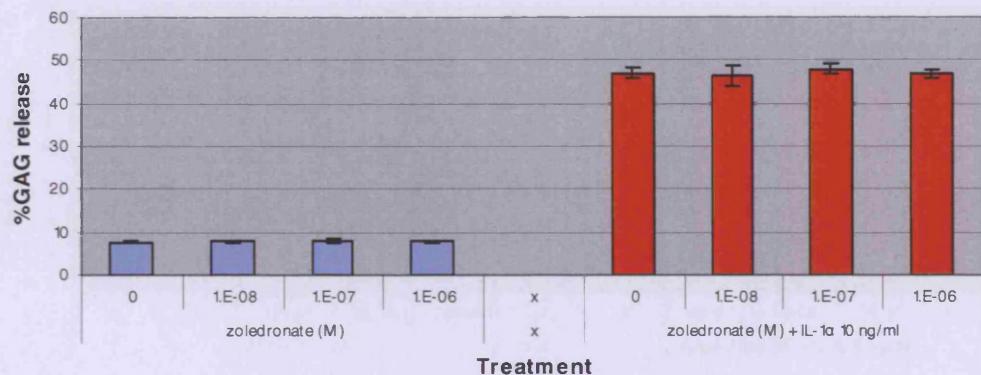
In one experiment IL-1 α 0.05 ng/ml was chosen as the stimulus since it had been previously shown that this concentration caused a sub-maximal stimulus of GAG release (Fig. 5.6) and it was considered that a gentler stimulus of GAG release might be more readily modulated by zoledronate. Unexpectedly, in controls, only a trend for increased GAG release was seen with IL-1 α 0.05 ng/ml compared to no IL-1 α ($P=0.06$). In cultures co-treated with IL-1 α 0.05 ng/ml, none of the zoledronate treatments tested (10^{-7} M to 10^{-4} M) was observed to alter GAG release compared to control.

Fig. 5.9. Exp. b.a3.2. Effects of zoledronate ($1.E-8$ M to $5.E-7$ M) on basal and IL-1 α -stimulated (10 ng/ml) GAG release in the bovine articular chondrocyte/ alginate bead culture model.



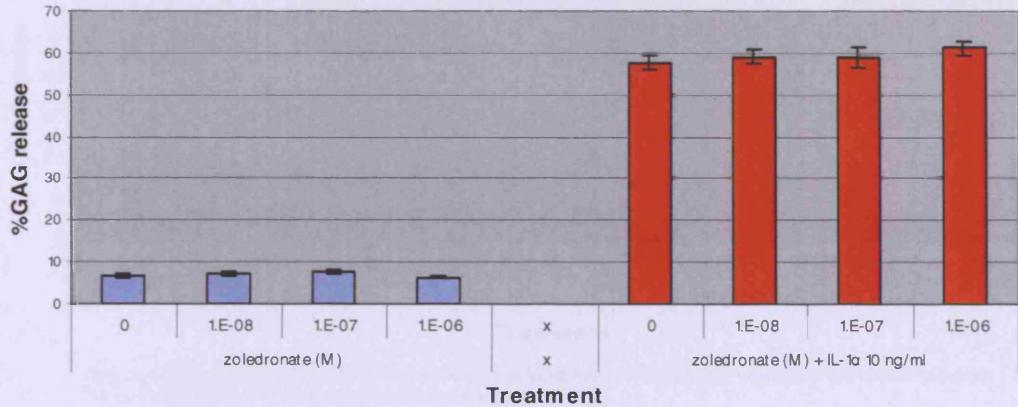
Beads containing bovine articular chondrocytes and an established matrix cultured in the presence of treatments for two days. Values for %GAG release from beads shown as means \pm SD ($n=6$).

Fig. 5.10. Exp. b.a4.2. Effects of zoledronate (1.E-8 M to 1.E-6 M) on basal and IL-1 α -stimulated (10 ng/ml) GAG release in the bovine articular chondrocyte/ alginate bead culture model.



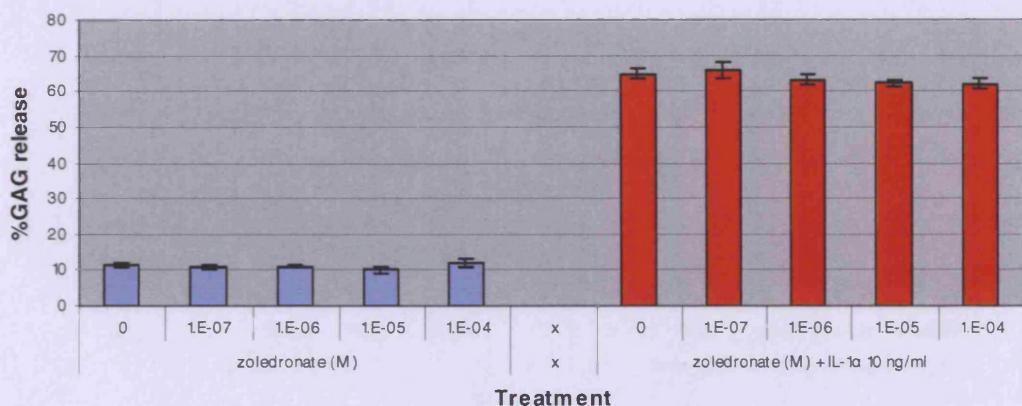
Beads containing bovine articular chondrocytes and an established matrix cultured in the presence of treatments for two days. Values for %GAG release from beads shown as means \pm SD (n=6).

Fig. 5.11. Exp. b.a4.4. Effects of zoledronate (1.E-8 M to 1.E-6 M) on basal and IL-1 α -stimulated (10 ng/ml) GAG release in the bovine articular chondrocyte/ alginate bead culture model.



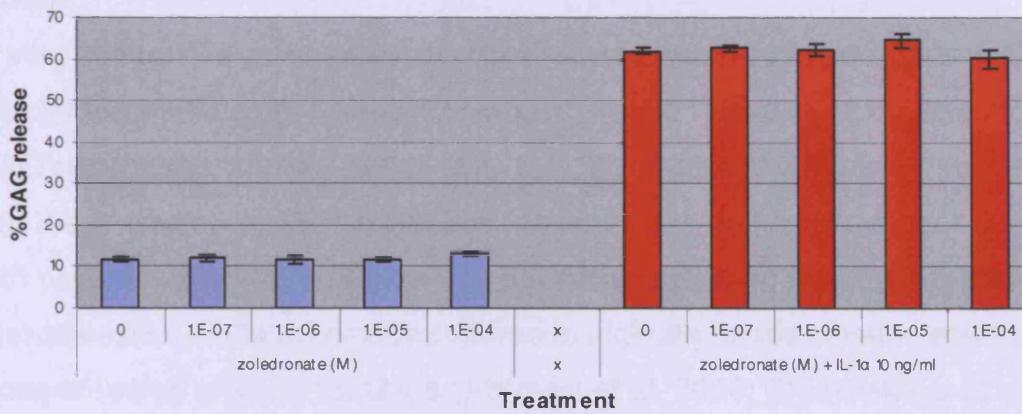
Beads containing bovine articular chondrocytes and an established matrix cultured in the presence of treatments for two days. Values for %GAG release from beads shown as means \pm SD (n=6).

Fig. 5.12. Exp. b.a5.3. Effects of zoledronate (1.E-7 M to 1.E-4 M) on basal and IL-1 α -stimulated (10 ng/ml) GAG release in the bovine articular chondrocyte/alginate bead culture model.



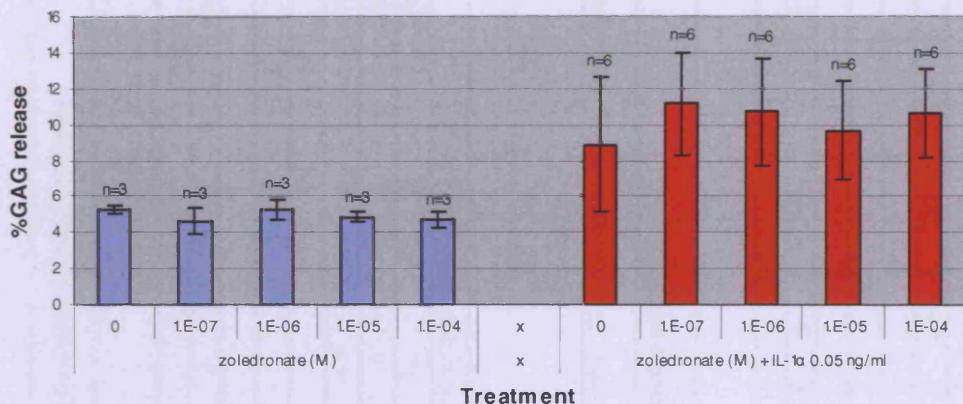
Beads containing bovine articular chondrocytes and an established matrix cultured in the presence of treatments for two days.
Values for %GAG release from beads shown as means \pm SD (n=6).

Fig. 5.13. Exp. b.a6.3. Effects of zoledronate (1.E-7 M to 1.E-4 M) on basal and IL-1 α -stimulated (10 ng/ml) GAG release in the bovine articular chondrocyte/alginate bead culture model.



Beads containing bovine articular chondrocytes and an established matrix cultured in the presence of treatments for two days.
Values for %GAG release from beads shown as means \pm SD (n=6).

Fig. 5.14. Exp. b.a7.6. Effects of zoledronate (1.E-7 M to 1.E-4 M) with and without IL-1 α 0.05 ng/ml co-treatment on GAG release in the bovine articular chondrocyte/ alginate bead culture model.



Beads containing bovine articular chondrocytes and an established matrix cultured in the presence of treatments for two days. Values for %GAG release from beads shown as means \pm SD (n=3 or n=6 as indicated on chart).

5.6 Discussion

Characteristics of the bovine articular chondrocyte/ alginate bead culture model

In vitro culture of bovine articular chondrocytes within alginate beads in serum-supplemented medium for periods ranging from 21 days to 42 days led to the GAG accumulation in the beads (Fig. 5.1). Synthesis of GAG is an indication that the chondrocyte phenotype was maintained in the culture model, consistent with previous detailed studies which have demonstrated that the cell and matrix characteristics of chondrocytes cultured in alginate beads closely resemble those of native articular cartilage (Almqvist et al. 2001; Chubinskaya et al. 2001; Guo et al. 1989; Hauselmann et al. 1992; Hauselmann et al. 1994; Hauselmann et al. 1996b; Petit et al. 1996), and supports the use of the culture system to investigate chondrocyte-matrix metabolism.

Table 5.4. Results summary from six experiments for the effects of zoledronate (1.E-8 M to 1.E-4 M) on basal GAG release from alginate beads containing chondrocytes and an established matrix.

Exp.	Mean %GAG release for control	Difference (Δ) in mean %GAG release between zoledronate and control; and significance level of the difference*											
		Zoledronate treatment concentration (M)											
		10 ⁻⁸		10 ⁻⁷		5x10 ⁻⁷		10 ⁻⁶		10 ⁻⁵		10 ⁻⁴	
		Δ	P value	Δ	P value	Δ	P value	Δ	P value	Δ	P value	Δ	P value
b.a3.2	16.7	-.2	.995	-.3	.973	+.2	.990						
b.a4.2	7.6	+.2	.782	+.3	.267			+.2	.619				
b.a4.4	6.9	+.3	.499	+.7	.034			-.5	.203				
b.a5.3	11.3			-.6	.589			-.3	.931	-1.3	.055	+.5	.715
b.a6.3	11.6			+.4	.725			-.1	.999	.0	>.999	+.1	.039
b.a7.6	5.2			-.6	.388			.0	>.999	-.4	.730	-.5	.338

* Since multiple experiments were performed the alpha level to indicate a significant difference between means was set at P=0.01 as described in section 2.3.2

Table 5.5. Results summary from five experiments for the effects of zoledronate (1.E-8 M to 1.E-4 M) on IL-1 α -stimulated (10 ng/ml) GAG release from alginate beads containing chondrocytes and an established matrix.

Exp.	Mean % GAG release for IL-1 α -stimulated control	Difference (Δ) in mean IL-1 α -stimulated %GAG release between zoledronate and control; and significance level of the difference*											
		Zoledronate treatment concentration (M)											
		10 ⁻⁸		10 ⁻⁷		5x10 ⁻⁷		10 ⁻⁶		10 ⁻⁵		10 ⁻⁴	
		Δ	P value	Δ	P value	Δ	P value	Δ	P value	Δ	P value	Δ	P value
b.a3.2	72.0	-.3	.970	-.6	.789	+.3	.967						
b.a4.2	47.0	-.5	.904	+.9	.586			-.2	.985				
b.a4.4	57.6	+.1.5	.383	+.1.4	.467			+.3.6	.011				
b.a5.3	64.8			+.1.1	.610			-.1.6	.288	-2.6	.033	-2.8	.022
b.a6.3	61.5			+.1.1	.663			+.9	.814	+.3.0	.012	-1.7	.284

* Since multiple experiments were performed the alpha level to indicate a significant difference between means was set at P=0.01 as described in section 2.3.2

Subsequent culture of alginate beads containing chondrocytes and an established matrix with IL-1 α stimulated GAG release from the beads. IL-1 α 10 ng/ml stimulated GAG release in a time-dependent fashion (Fig. 5.2), which is consistent with a previous study that found a similar relationship for IL-1 β stimulation in the same culture system (Beekman et al. 1998). In the present studies, the greatest separation between basal and IL-1 α stimulated GAG release occurred with two-day culture (Fig. 5.2) leading to the selection of this stimulation period in all subsequent experiments. IL-1 α at the concentration of 10 ng/ml was used in nearly all experiments and, consistently, this stimulus was found to increase GAG release (Fig. 5.7). These observed effects of IL-1 α 10 ng/ml support its use in the model to investigate chondrocyte-mediated proteoglycan degradation.

Later on in the studies, GAG release as a function of IL-1 α concentration in the model was explored to examine the possibility that GAG release might saturate at higher IL-1 α concentrations. Across three experiments, the IL-1 α concentration range evaluated was 0.05 ng/ml to 20 ng/ml. Surprisingly, though all IL-1 α concentrations tested did stimulate GAG release, the highest cytokine concentrations did not produce maximal stimulatory effects (Figs. 5.3 to 5.6). It is possible that chondrocytes may become initially desensitised to IL-1 α at high ligand concentrations since human dermal fibroblasts, another type of connective tissue cell, have been found to transiently down-regulate IL-1 receptors on exposure to IL-1 α 5 ng/ml (though receptor expression then up-regulated on longer-term exposure to the cytokine) (Akahoshi et al. 1988). Alternatively, there may be a mild cytotoxic effect at higher IL-1 α concentrations, as is suggested by one of the viability experiments described in Chapter 3 in which IL-1 α 10 ng/ml treatment caused a 3.1% reduction in chondrocyte viability (Table 3.3). Clarification of these possible mechanisms would require further study.

IL-1 α 0.05 ng/ml, which was the lowest IL-1 α concentration evaluated, also produced a sub-maximal stimulatory effect on GAG release (Figs. 5.6). Taken

together, the results suggest a biphasic relationship: at lower IL-1 α concentrations the amounts of GAG release increase with cytokine concentration up to a peak beyond which higher IL-1 α concentrations are less stimulatory for GAG release.

For evaluating the effects of zoledronate on GAG release in the culture model, it was considered that a milder stimulus of proteoglycan degradation might be more readily modulated by the bisphosphonate. Thus, IL-1 α 0.05 ng/ml was selected as a sub-maximal stimulus for the culture model in one experiment that tested the general hypothesis. However, in this experiment, IL-1 α 0.05 ng/ml did not stimulate GAG release significantly and, in addition, between-sample variation for GAG release was high (CV=42.2% for GAG release with IL-1 α 0.05 ng/ml). The level of variability was unexpected since the cell source was standardised between samples, and it is assumed that variability was introduced during execution of the experimental methods. Additional work is needed on the model to identify relatively low concentrations of IL-1 α that mildly stimulate GAG release and, in further experiments, particular attention should be given to meticulous experimental technique.

In terms of within-experiment reproducibility for GAG release stimulated by IL-1 α 10 ng/ml, the culture model performed well. The mean intra-experimental CV for this measure was 3.6%, comparing favourably with that seen in the cartilage explant model (mean CV 18.4%; Table 4.3). Thus, of the two culture models, the bovine articular chondrocyte/ alginate bead model possesses greater sensitivity for detecting treatment effects on GAG release stimulated by IL-1 α 10 ng/ml.

Interestingly, a recurring theme seen in the chondrocyte/ alginate bead culture system was inter-experimental variation. Firstly, the rate of bead GAG accumulation during the matrix-establishing culture period appeared to differ strikingly between experiments (Fig. 5.1), even in the absence of formal statistical comparisons which were not performed because the time points for

measuring GAG accumulation were not uniform across studies. Secondly, the amounts of GAG released from the beads (both basal and IL-1 α -stimulated) varied widely between experiments (Figs. 5.7 and 5.8). Thirdly, the IL-1 α concentration that was found to cause maximal GAG release was not uniform (IL-1 α 2 ng/ml in two experiments and IL-1 α 0.5 ng/ml in the third). Fourthly, IL-1 α 0.05 ng/ml increased GAG release in one experiment but only led to a trend for increased GAG release in another experiment (Fig. 5.8).

Variability between experiments could have arisen from biological differences in the chondrocytes and/or differences in culture technique. The use of 10% FCS to supplement the culture medium in one experiment (b.a3) may have led to a reduced rate of bead GAG accumulation compared other experiments in which 20% FCS was used since FCS is a known stimulus of proteoglycan synthesis in bovine articular chondrocytes (van Susante et al. 2000). In addition, the culture duration during which matrix was established in the beads prior to IL-1 α stimulation was different between experiments (it ranged from 21 days to 42 days). Previous studies indicate that aspects of chondrocyte metabolism, such as the rates of cell proliferation and GAG synthesis, can vary with culture duration (Akmal et al. 2006). Catabolic responses of chondrocytes might also change according to in vitro culture duration, which would offer some explanation for the observed inter-experimental variation in bead GAG release.

Alternatively, inter-experimental variation may have reflected inter-animal differences in chondrocyte proteoglycan metabolism, though the use of at least two animals to source chondrocytes in most experiments is likely to have somewhat averaged-out such differences between experiments (Tables 5.2 and 5.3). Since older age has been found to be associated with decreased responsiveness of articular cartilage to the effects of IL-1 (Hauselmann et al. 1996a; Little et al. 1999; MacDonald et al. 1992), the variation in animal age in the present study (known ages of animals ranged from 13 months to 29 months) may have been significant for between-experiment differences.

Investigating the marked variation in chondrocyte metabolism seen in the present studies should identify model parameters to be standardised in further experiments and, in addition, could provide further insight into the role of IL-1 in OA and improve understanding of OA aetio-pathogenesis. Effects of differences in culture technique could be examined in a standardised cell line. Biological differences could be explored using standardised methodology for the culture model whilst varying the cell source, for example examining the responsiveness to IL-1 α of chondrocytes from various regions of the articular surface defined according to susceptibility to cartilage degeneration.

Effects of zoledronate on chondrocyte-mediated proteoglycan degradation

In the studies, zoledronate treatments at concentrations ranging from 10⁻⁸ M to 10⁻⁴ M were not observed to have effects on unstimulated GAG release or GAG release stimulated by IL-1 α 10 ng/ml in the bovine articular chondrocyte/alginate bead culture model (Tables 5.4 and 5.5). At specific zoledronate concentrations, sporadic trends for treatment effects were seen (both increases and decreases with effect sizes ranging from 4% to 10.6% compared to control), but since no trends were reproduced between experiments, they are not considered to be significant. Taken together, the negative findings can be interpreted to be biologically relevant for bovine animals in general as chondrocytes were sourced from different animals between experiments.

In order to explore the possibility that zoledronate might only have a moderate modulatory effect on IL-1 α -stimulated proteoglycan degradation, an attempt was made to investigate effects of the bisphosphonate in the culture model but with a milder stimulus of GAG release. However, as described above (this section), a mild stimulus was not successfully identified in the present studies and further work is needed on the model in order to pursue this line of investigation. Ultimately, finding positive though mild modulatory effects on proteoglycan degradation may translate to clinical disease modification if the effects are

sustained and, thereby, lead to increases in cartilage proteoglycan content over time.

Overall, no effects of zoledronate were found to support the general hypothesis. On the other hand, there were also no observable detrimental effects that would exclude zoledronate as a disease-modifying treatment for OA through an action on cartilage metabolism.

5.7 Conclusions

- Bovine articular chondrocytes that are cultured in alginate beads establish GAG in the beads. Subsequent exposure of these beads to IL-1 α 10 ng/ml stimulates GAG release with excellent between-sample reproducibility. Thus, the culture system provides an *in vitro* model of chondrocyte-matrix catabolism for sensitively investigating treatment effects on proteoglycan degradation.
- Generally, IL-1 α has stimulatory effects on GAG release in the model. GAG release as a function of IL-1 α concentration appears to be biphasic with positive correlation between GAG release and IL-1 α concentration at lower cytokine concentrations and negative correlation at higher IL-1 α concentrations.
- Between experiments, there were high levels of variation in the rate of GAG accumulation in alginate beads and the subsequent release of GAG from the beads (with and without IL-1 α stimulation). This inter-experimental variation could have arisen from biological differences in the chondrocytes and/or differences in culture technique. Further study of inter-animal variation in chondrocyte-mediated proteoglycan metabolism in the culture model could yield important insight into aetio-pathogenesis of cartilage degradation in OA.

- Zoledronate treatments 10^{-8} M to 10^{-4} M were not observed to have effects on GAG release stimulated by IL-1 α 10 ng/ml from alginate beads containing matrix established by bovine articular chondrocytes. Thus, no evidence was found to support the hypothesis that the bisphosphonate can decrease the loss of cartilage glycosaminoglycan during the osteoarthritic process by reducing chondrocyte-mediated proteoglycan degradation.

Chapter 6. General discussion

6.1 Aim of studies

The studies described in this thesis have explored the concept that zoledronate can alter the disease course in OA by testing the hypothesis that the bisphosphonate can modify cartilage metabolism to reduce the loss of cartilage proteoglycan during the OA disease process.

6.2 In vitro models employed in experiments

Effects of zoledronate on proteoglycan synthesis and degradation, that is both sides of the metabolic balance, were examined in vitro in models of chondrocyte and cartilage metabolism, in which IL-1 α was used to stimulate "OA-like" metabolic change. The two culture models employed, bovine articular cartilage explant culture model and bovine articular chondrocyte/ alginate bead culture model, were found to be effective for investigating effects on IL-1 α -stimulated proteoglycan degradation (measured as tissue GAG release). In addition, the chondrocyte/ alginate bead model proved useful for evaluating effects of zoledronate on chondrocyte proteoglycan synthesis, as well as potential adverse effects on chondrocyte viability and proliferation.

In relation to the bovine cartilage explant culture model, an interesting observation was marked intra-joint variability in IL-1 α -stimulated GAG release in cartilage. Heterogeneity in the biochemical composition of articular cartilage across the joint surface is well known. Previous reports indicate that GAG content (Brama et al. 2000), chondroitin sulphate sulphation pattern (Bayliss et al. 1999) and aggrecanase-generated aggrecan fragment distribution (Bayliss et al. 2001) differ according to the site sampled. In addition, susceptibility to IL-1 β -stimulated GAG release has been found to vary in cartilage from different anatomical locations in the human OA knee joint (Barakat et al. 2002). Further work to examine if the susceptibility of articular cartilage to IL-1 α -stimulated proteoglycan degradation relates to cartilage sites that develop OA change

within a joint could provide additional insight into the mechanisms behind focal cartilage loss in OA.

The levels of intra-experimental variability in IL-1 α -stimulated GAG release for the cartilage explant model posed a problem for experimental design.

Unfeasibly large sample numbers would have been required in the model to achieve adequate power to detect small-sized treatment effects (e.g. from sample size calculations: up to 102 samples per treatment group needed to detect a 10% effect size with 80% power). Another culture system, the chondrocyte/ alginate bead model which involved culturing a “cartilage-like” tissue in vitro, demonstrated much less between-sample variability within experiments and was also used to investigate the effects of zoledronate on IL-1 α -stimulated GAG release. It is likely that the improved levels of variability resulted from standardisation of the cell source for samples; alginate beads for all samples within experiments were formed using the same homogenised pool of cells in which any topographical differences in chondrocyte metabolism would have been averaged out. In further studies, the model could be used to explore possible topographical variation in chondrocyte metabolism by examining response to IL-1 α in cells sourced from defined areas of an articular surface of a single joint.

In both culture models, marked inter-experimental variability was encountered for the amounts of tissue GAG released, an unexpected finding since sources for cartilage or chondrocytes were relatively standardised across experiments. The studies for this project were not designed to examine the basis for this variability but one possible explanation is metabolic variation between animals. Additional work to assess cartilage and chondrocyte proteoglycan metabolism in relation to animal characteristics (e.g. age, sex and weight) could provide new information on the link between aetiology and pathogenesis for cartilage proteoglycan loss in OA.

6.3 Effects of zoledronate in the models of chondrocyte and cartilage metabolism

The usefulness of any potential treatment for OA with proposed effects on cartilage metabolism is in part defined by an absence of toxic effects on chondrocytes. Based on the assessment of effects on basic parameters of chondrocyte metabolism (i.e. cell viability, proliferation and proteoglycan synthesis), zoledronate concentrations $\leq 10^{-5}$ M appear safe. However, zoledronate 10^{-4} M is clearly detrimental to bovine chondrocytes from the MCP joint. It would be advisable to avoid exposing cartilage to zoledronate concentrations $\geq 10^{-4}$ M during clinical use, though pharmacokinetic data have yet to be reported on cartilage levels achieved with current dosing regimens. Furthermore, toxicity studies would need to be repeated using human chondrocytes in order to form firm conclusions.

Across the series of studies, no evidence was demonstrated to support the general hypothesis that zoledronate modifies cartilage metabolism to reduce the loss of cartilage glycosaminoglycan during the OA process. Specifically, no enhancing effects were observed with zoledronate 10^{-10} M to 10^{-4} M on proteoglycan synthesis in the chondrocyte/ alginate bead culture model and, furthermore, no modulating effects on proteoglycan degradation were seen with zoledronate 10^{-10} M to 10^{-5} M in the cartilage explant culture model or with zoledronate 10^{-8} M to 10^{-4} M in the chondrocyte/ alginate bead culture model.

Prior to performing the studies, it had been considered that inhibition of MMP or aggrecanase activity was a plausible biochemical mechanism for an inhibitory effect of zoledronate on proteoglycan degradation. However, proteinase inhibition requires high zoledronate concentrations, in the range 2.5×10^{-5} M to 10^{-3} M (Heikkila et al. 2002; Teronen et al. 1999), and in the present studies only zoledronate treatment at concentrations $\leq 10^{-5}$ M appeared safe for bovine articular chondrocytes. Thus, at least for bovine articular cartilage, there is no therapeutic window for zoledronate to modulate proteoglycan degradation through proteinase inhibition.

Overall, the negative findings for the general hypothesis may be interpreted in three ways: (i) the general hypothesis has not been adequately tested due to limitations of the experimental approach; (ii) the overall concept of zoledronate as a disease-modifying treatment in OA is valid but its activity arises from a mechanism of action other than a direct effect on cartilage proteoglycan metabolism; or (iii) zoledronate does not have OA disease-modifying properties.

6.4 Limitations of the studies

The present studies have looked for direct effects of zoledronate on cartilage or chondrocyte metabolism in vitro. Several limitations to the experimental approach are apparent.

Firstly, some characteristics of the in vitro culture systems that were employed in the experiments indicate that these models are less than accurate representations of in vivo metabolism:

- a) **Regulation of cartilage metabolism in vitro and in vivo:** The 10 ng/ml concentration of IL-1 α employed in the models to stimulate proteoglycan degradation is nearly 100-fold higher than IL-1 levels (albeit IL-1 β rather than IL-1 α) seen in synovial fluid of knee OA joints (Westacott et al. 1990). Furthermore, the use of a single cytokine to stimulate metabolism is simplistic compared to the in vivo setting, in which regulation of metabolism is governed by various other cytokines, biochemical factors and mechanical factors that, in addition, can interact with one another (discussed in section 1.2.3.6).

Several reports indicate that In vitro culture techniques introduce changes to metabolism in cartilage and chondrocytes. In vitro culture of cartilage explants or chondrocytes alters proteinase expression patterns, in comparison to those found in fresh cartilage tissue extracts (Bau et al.

2002; Flannery et al. 1999). In addition, the cutting of articular cartilage releases tissue bound basic fibroblastic growth factor (Vincent et al. 2002) (a proposed extracellular mechanotransducer (Vincent and Saklatvala 2006)), increases cell proliferation and matrix synthesis at the wound edge (Redman et al. 2004), and induces IL-1 (Gruber et al. 2004). Furthermore, the initial removal of the peri-cellular matrix during chondrocyte isolation in the chondrocyte/ alginate bead culture model is likely alter cell metabolism since this domain of matrix is thought to be a transducer of biomechanical and biochemical signals (Guilak et al. 2006). However, a pre-culture period was employed to stabilise cartilage or chondrocytes prior to treatment culture in all but one of the experiments of the present studies and its use is likely to have minimised acute effects of tissue processing.

b) **Differences in rate of change:** The rate of matrix change observed in the in vitro models is more rapid than that in vivo. IL-1 α stimulation in vitro led to up to 62.9% of the proteoglycan being released from cartilage over a four-day culture period. In comparison, the proteoglycan content in OA cartilage from OA hips removed at arthroplasty has been reported to be 52% less than that in cartilage from normal control hips (Byers et al. 1977). Though effect sizes are similar, the time course for pathological change in the OA hips must have been over several years, implying that quantitatively small but sustained alterations to metabolism could be significant for cartilage proteoglycan loss in vivo.

Secondly, the specific use of young adult, healthy cartilage from the metacarpal articular surfaces of bovine MCP joints in the studies somewhat limits interpretation of the results. Cartilage composition and metabolism varies between species (Cawston et al. 1998; Hughes et al. 1998), in different joints (Eger et al. 2002) and in relation to age (Hardingham 2004). Thus, the negative findings from the present studies should be interpreted cautiously in the context of the heterogeneous nature of cartilage metabolism. In particular, the use of

results derived from present studies to understand the mechanisms behind the reductions in cartilage degradation with zoledronate treatment seen in rabbits with experimentally-induced OA (Muehleman et al. 2002) is limited by the possibility of inter-species differences in cartilage metabolism.

Other study limitations relate to potential zoledronate treatment effects in the model that were not addressed. An attempt had been made to examine treatment effects on proteoglycan degradation stimulated by a milder stimulus (i.e. IL-1 α at the 0.05 ng/ml concentration rather than 10 ng/ml) but conclusions were not drawn because the culture model did not perform as expected. Preventative or delayed treatment effects also have yet to be examined. The maximum duration of zoledronate treatment in the present studies was four days and the bisphosphonate was added at the same time as the IL-1 α stimulus. In comparison, the zoledronate treatment period that led to reductions in cartilage degradation in the animal OA model commenced 24 hours before induction of cartilage damage and then continued for 28 days or 56 days prior to assessment (Muehleman et al. 2002), raising the possibility of preventative or delayed treatment effects. Furthermore, the inhibitory effects of etidronate and clodronate on mono-nuclear cell factor-stimulated collagenase production by chondrocytes have been found to be more pronounced when cells were pre-treated with the bisphosphonates for several days than when they were added at the same time as the stimulating factor (McGuire et al. 1982), suggesting that bisphosphonates in general may have preventative or delayed effects on chondrocyte metabolism.

6.5 Zoledronate as a disease-modifying treatment in OA: possible mechanisms of action other than a direct effect on cartilage proteoglycan metabolism

An alternative target for zoledronate in cartilage is the metabolism of type II collagen. Observed reductions in urinary CTX-II in Paget's disease patients treated with zoledronate (Garnero et al. 2001a) point toward an inhibitory effect

on type II collagen degradation. As discussed above, zoledronate can inhibit MMP proteolytic activity, providing a biochemical mechanism, but there does not appear to be a therapeutic window for MMP inhibition in cartilage without adverse effects on chondrocytes.

Beyond cartilage, possible mechanisms of action for zoledronate as a disease-modifying treatment in OA include effects on any one of the other joint tissues involved in pathogenesis. In particular, subchondral bone is a plausible target and there has been considerable recent interest in the potential for agents with anti-resorptive effects on bone as therapies in OA (Abramson and Honig 2007; Burr 2004; Spector 2003). Numerous studies have reported positive effects with various bisphosphonates or calcitonin (another anti-resorptive) in animal OA models (Agnello et al. 2005; Doschak et al. 2004; Hayami et al. 2004; Manicourt et al. 1999; Meyer et al. 2001a; Meyer et al. 2001b; Muehleman et al. 2002; Papaioannou et al. 2007; Sondergaard et al. 2007). In addition, clinical trials have evaluated risedronate treatment in OA patients. Encouraging results were found in an initial study of 284 patients with knee OA (Spector et al. 2005) but a subsequent larger study of nearly 2500 knee OA patients did not find clinical improvements or reductions in joint-space narrowing with risedronate (Bingham, III et al. 2006). It has been suggested that the latter study was under-powered for primary outcome measures due to low numbers of OA patients demonstrating disease progression (Abramson and Honig 2007). An alternative explanation for the discrepant results for bisphosphonate efficacy between the human and animal studies is the difference in treatment timing in relation to the OA disease process. In many animal studies, anti-resorptive treatment commenced around the time that experimental OA was induced (Agnello et al. 2005; Doschak et al. 2004; Hayami et al. 2004; Manicourt et al. 1999; Meyer et al. 2001a; Meyer et al. 2001b; Muehleman et al. 2002; Papaioannou et al. 2007; Sondergaard et al. 2007) whereas in the human study, the bisphosphonate was given to patients with established knee OA (Bingham, III et al. 2006). Thus, it may be that inhibiting bone resorption to alter OA pathogenesis may be only effective if inhibition occurs during the early stages of the OA process.

It is interesting to speculate how reducing bone resorption might affect the OA disease process. In OA, articular surface deformity and limb malalignment are thought to arise partly from collapse of weakened sub-articular cancellous bone (Buckland-Wright 2004). Peri-articular bone loss, which is seen in the OA joint (Kannus et al. 1992; Karvonen et al. 1998; Leppala et al. 1999), could predispose to structural failure. Prevention of bone loss with anti-resorptive therapies may, therefore, ultimately result in improved mechanical characteristics of the OA joint. Reducing osteophyte formation, as has been observed for alendronate in an OA animal model (Hayami et al. 2004), is another way that inhibition of bone resorption could structurally modify the OA joint. However, this may not be ultimately beneficial because osteophytes may in fact improve joint stability (Pottenger et al. 1990).

An effect on proposed biochemical interactions between subchondral bone and articular cartilage is an alternative mechanistic explanation for a bone effect. Through biochemical interactions, abnormal osteoblasts from OA subchondral bone may be involved in promoting proteoglycan loss in adjacent articular cartilage (Sanchez et al. 2005; Westacott et al. 1997). If activation of abnormal osteoblasts occurs as part of the bone remodelling process, then inhibiting bone turnover in OA subchondral bone with anti-resorptive therapies may lead indirectly to improved proteoglycan retention in articular cartilage.

Lastly, increases in systemic BMD may have an effect on the OA process. High BMD has been reported as a risk factor for incident OA whereas low BMD is a risk factor for progressive disease in patients with established OA (Hart et al. 2002; Zhang et al. 2000). If these risk factors prove to be causal in nature, then increasing BMD with anti-resorptive therapies would be expected to increase incident OA but reduce progressive OA.

A caveat to the hypothesis that inhibiting bone remodelling modulates the OA disease process is the finding that treatment efficacy for a range of bisphosphonates in the Duncan-Hartley guinea pig model of OA did not relate to

the anti-resorptive potency of the bisphosphonates (Meyer et al. 2001a). Additional work is needed to examine the role of bone remodelling in OA and the efficacy of inhibiting bone turnover for modifying the disease process.

6.6 Is there a role for zoledronate as a disease-modifying treatment in OA?

Overall the findings from these present studies do not suggest a role for zoledronate as a disease-modifying treatment in OA through an effect on cartilage proteoglycan metabolism. However, as discussed above, there are limitations to the present studies and other mechanisms of action remain unexplored. Further work to address these issues may yet find evidence for a mechanism of action and, in addition, provide insight into the pathophysiological relationship between subchondral bone and articular cartilage in the OA joint.

Chapter 7. General conclusions

- In vitro culture of bovine articular cartilage explants with and without IL-1 α 10ng/ml stimulation provides a model for investigating cartilage proteoglycan degradation.
- In vitro culture of bovine articular chondrocytes in alginate beads provides a model for evaluating chondrocyte viability, proliferation and proteoglycan synthesis. In addition, following a period in culture to establish matrix in the alginate beads, subsequent culture of the beads with and without IL-1 α 10 ng/ml provides a model for investigating chondrocyte-mediated proteoglycan degradation. Tissue GAG release is less variable in the chondrocyte/ alginate bead culture model than in the cartilage explant culture model. Thus, treatment effects on GAG release can be detected more sensitively in the former model.
- Zoledronate treatments at concentrations $\leq 10^{-5}$ M generally appear safe for bovine articular chondrocytes. However, zoledronate 10^{-4} M has adverse effects on chondrocyte viability, proliferation and proteoglycan synthesis.
- No evidence was demonstrated that short-term treatment with zoledronate $\leq 10^{-4}$ M enhances chondrocyte proteoglycan synthesis or reduces IL-1 α -stimulated chondrocyte-mediated proteoglycan degradation. Thus, the results do not support the hypothesis that zoledronate has a role as a disease-modifying treatment in OA through an effect on cartilage to reduce the pathological loss of cartilage proteoglycan.
- Preventative or delayed zoledronate treatment effects on cartilage proteoglycan metabolism remain unaddressed. In addition, it is possible that zoledronate can modify the OA disease process through effects on cartilage

type II collagen metabolism or effects on another joint tissue, such as subchondral bone.

Chapter 8. Further work

Optimising the models of cartilage or chondrocyte-matrix catabolism

Adjustments to parameters of the in vitro chondrocyte or cartilage culture systems used in these present studies could be made to improve their accuracy as models of in vivo metabolism. The OA disease process is slow and it can take many years before pathology becomes evident. The chronic nature to pathogenesis is seen clearly in patients with trauma to the knee causing injury to the anterior cruciate ligament or meniscus, in whom the first radiographic signs of OA appeared on average about 10 years after the injury (Roos et al. 1995). It follows that even minor imbalances to degradation and repair in cartilage may be important for cartilage loss if they are sustained over long periods of time. Studies to characterise proteoglycan degradation as a function of time and IL-1 α concentration could be followed by the development of accurate in vitro models of in vivo cartilage metabolism in which GAG release stimulated by a gentle IL-1 α stimulus can be observed over long term culture.

In view of the marked inter-experimental variation in proteoglycan metabolism seen in the models of the present studies, future experiments should be performed with standardised cell / tissue sources (e.g. a single pool of cells or an immortalised chondrocyte cell line) and standardised culture techniques.

Additional testing of zoledronate for effects on cartilage proteoglycan metabolism

Further work should examine preventative and delayed treatment effects of zoledronate on cartilage proteoglycan synthesis and degradation since these have not been addressed in these present studies. In addition, subsequent experiments could use cartilage or chondrocytes from rabbits instead of a bovine source in the culture models. Findings from such experiments would be informative for understanding the mechanisms behind the beneficial effects of zoledronate treatment in rabbits with experimentally-induced OA (Muehleman et

al. 2002) without being limited by inter-species differences in cartilage or chondrocyte metabolism.

Examining other mechanisms of action for zoledronate as a disease-modifying treatment in OA

Effects of zoledronate on cartilage type II collagen synthesis or degradation are unknown. In order to address this, an alternative culture model to those used in the present studies would be needed since little or no collagen degradation is induced by IL-1 in bovine articular cartilage explants (Caterson et al. 2000). One candidate model is the co-stimulation of collagen degradation in bovine nasal cartilage or human articular cartilage with IL-1 α and oncostatin M (Cawston et al. 1998).

More information about potential target tissues for zoledronate could be derived in animal OA models. Studies to reproduce the treatment effects previously demonstrated a rabbit OA model (Muehleman et al. 2002) could examine, in addition, the localisation of zoledronate within the joint. The absence of zoledronate in any particular joint tissue would exclude it from being a target tissue whereas the presence of the bisphosphonate would indicate a possible target tissue. These findings would be useful for directing further studies of target tissues to elucidate underlying metabolic mechanisms.

Overall, the continued search for disease-modifying treatments for OA may lead not only to the identification of clinically useful treatments but, if underlying mechanisms are known, also add to the understanding of OA pathogenesis.

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