BISPHOSPHONATES IN THE TREATMENT OF RHEUMATOID ARTHRITIS

With special reference to collagen degradation

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ACADEMIC DISSERTATION

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ABBREVIATIONS

ACR	American College of Rheumatology
$\alpha_2 M$	α_2-macroglobulin
ANOVA	analysis of variance
AP-1	activating protein-1
BM	basement membrane
BMD	bone mineral density
BP	bisphosphonate
CRP	C-reactive protein
CTx	carboxyterminal cross-linked peptide of type I collagen
DAS	disease activity score
DMARD	disease modifying antirheumatic drug
ELISA	enzyme linked immunoassay
EMSA	Electrophoretic mobility shift assay
ESR	erythrocyte sedimentation rate
ICTP	cross-linked carboxyterminal telopeptide of type I collagen
IGF	insulin-like growth factor
IL	interleukin
LPS	lipopolysaccharide
PBS	phosphate buffered saline
PMN	polymorphonuclear leukocyte
M-CSF	macrophage colony stimulating factor
MCP-3	monocyte chemoattractant protein-3
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-B
NTx	cross-linked aminoterminal telopeptide of type I collagen
OA	osteoarthritis
OPG	osteoprotegerin
PAGE	polyacrylamide gel electrophoresis
PICP	carboxyterminal propeptide of type I procollagen
PINP	aminoterminal propeptide of type I procollagen
PTH	parathyroid hormone
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor -kappa B ligand
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
TFG	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
TRAP	tartrate-resistant acidic phophatase
VAS	visual analogue scale

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerals I–V:

I   Valleala H, Laitinen K, Pylkkänen L, Konttinen YT, Friman C. Clinical and biochemical response to single infusion of clodronate in active rheumatoid arthritis. Inflamm Res 2001; 50: 598–601.

II   Valleala H, Teronen O, Friman C, Sorsa T, Solovieva S, Konttinen Y T. Inhibition of collagenase by a bisphosphonate-group drug in rheumatoid arthritis patients. J Rheumatol 2000; 27: 1570–2.

III   Valleala H, Laasonen L, Koivula M-K, Mandelin J, Friman C, Juha Risteli, Konttinen YT. Two year, randomized, controlled trial of rheumatoid arthritis with etidronate: Changes in serum aminoterminal telopeptides correlate with the radiographic progression of the disease. J Rheumatol 2003; 30: 468–73.

IV   Valleala H, Mandelin J, Laasonen L, Koivula M-K, Risteli J, Konttinen YT. Effect of cyclical intermittent etidronate therapy on circulating osteoprotegerin levels in rheumatoid arthritis patients. Eur J Endocrinol 2003; 148: 527–30.

V   Valleala H, Hanemaaijer R, Mandelin J, Salminen A, Teronen O, Mönkkönen J, Konttinen YT. Regulation of MMP-9 (gelatinase B) in activated human monocyte/macrophages by two different types of bisphosphonates. Life Sciences 2003; 73: 2413–20.

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1. ABSTRACT

Rheumatoid arthritis (RA) is characterized by chronic inflammation in multiple joints and concomitant destruction of cartilage and bone. Cells of the monocyte/macropahge lineage play a crucial role in both the inflammatory process and tissue destruction. The main interest in the present study was to evaluate the antiarthritic and anticollagenolytic properties of two bisphosphonates (BP), clodronate and etidronate, in RA patients, and to examine the relationship of structural damage and serum biochemical markers of bone metabolism. We also compared the effects of clodronate and pamidronate on matrix metalloproteinase (MMP)-9 production by activated human monocyte/macropahges *in vitro.*

26 patients with active RA were randomly allocated to receive either a single i.v. infusion of 600 mg clodronate or placebo. Clinical assessments were carried out and serum and salivary samples were collected weekly during the following three weeks. Salivary samples were studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for collagenolytic activity and by Western blotting. In a 2 year randomized trial of etidronate including 40 patient with early RA, van der Heijde modified Sharp's method was used to evaluate the progression of radiographic joint damage. Radioimmunoassays and enzyme linked immunoassays (ELISA) were used for measurement of serum markers of type I collagen metabolism and osteoprotegerin. MMP-9 expression in and secretion from stimulated human monocyte/macrophages were measured using quantitative reverse transcriptase – polymerase chain reaction (RT-PCR) and ELISA, respectively.

Single infusion of clodronate did not suppress the markers of disease activity. However, three weeks after the drug infusion the salivary collagenase activity against soluble collagen type I monomers was found to be lower in the clodronate treated patients compared to controls. Concomitant Western blots using polyclonal antibodies specific to MMP-8, MMP-1 and MMP-13 revealed that neutrophil-type collagenase, MMP-8, was the major collagenase present in saliva of RA patients. Cyclical etidronate did not retard the progression of radiographic joint damage. Changes in serum aminoterminal telopeptides of type I collagen (NTx) were significantly associated with the development of focal articular bone erosions. Clodronate dose-dependently downregulated MMP-9 secretion by cultured human monocytes, whereas pamidronate significantly increased MMP-9 mRNA.

Significant association between change in serum NTx and worsening of erosion score provides biochemical evidence for the concept that osteoclast is the principal cell type responsible for the local bone resorption in RA, and suggests that monitoring changes in serum NTx may be useful to predict efficacy of treatment on progression. Lack of suppressive effects of BP treatment on synovial inflammation may be related to the low circulating levels achieved with doses used in the treatment of osteoporosis. Radiographic scores continued to deteriorate in the 2 year study irrespective of treatment which may be due to the fact that the least potent BP, i.e. etidronate, was used as an active drug. Clodronate is more potent and, unlike etidronate, can be administered continuously. Downregulation of leukocyte-derived MMPs, as suggested in this study, could represent an additional antiarthritic mechanism of clodronate. Upregulation of MMP-9 expression in activated human monocytes by pamidronate could, at least in part, explain why this drug did not demonstrate suppressive effects on focal bone erosions in a previous RA trial. Our group has now started a long term trial to evaluate the ability of clodronate to prevent structural damage in RA.

2. INTRODUCTION

Rheumatoid arthritis (RA) is chronic inflammatory disorder with systemic features and joint involvement, resulting in an erosive synovitis, cartilage degradation, and joint destruction. Structural damage to joint is a predictor of long-term outcome and it contributes over time to functional decline, disability and need for major surgical procedures (Scott *et al*., 2000). Protecting bone and articular cartilage from damage, consequently, has major potential both therapeutically and economically.

Studies of tissue sections from sites of bone erosions in RA have identified multinucleated cells with the phenotype of osteoclasts in bone resorbing lacunae, suggesting that osteoclasts mediate this pathologic bone loss. Osteoclast is derived from monocyte/macrophage precursors and is uniquely adapted to its resorbing activity through expression of distinct integrins as well as enzymes such as matrix metalloproteinases (MMPs) and cathepsin K (Goldring and Gravallese, 2000). Among them, cathepsin K is considered to be the most important enzyme in bone resorption because of its collagenolytic activity within a broad range pH optimum and selective expression in osteoclasts (Okada, 2001).

MMPs are overexpressed in tissues derived from rheumatoid joints (Konttinen *et al*., 1999) and the correlation of radiographic joint damage with a marker for MMP-mediated collagen degradation, i.e. the assay for cross-linked carboxy-terminal telopeptide of type I collagen (ICTP), suggests a role for MMPs in joint destruction (Hakala *et al*.,1993; Sassi *et al*., 2000). However, the clinical trials of broad spectrum MMP inhibitors in patients with joint disease have so far been unsuccessful (Catterall and Gavston, 2003). This underlines the need to identify the relevant MMPs in the joint destructive process and the development of selective inhibitors.

Bisphosphonates (BP) are carbon-substituted pyrophosphate analogues that have become the treatment of choice for a variety of bone diseases in which excessive osteoclast activity is an important pathological feature, including osteoporosis, Paget's disease and malignant bone disease (Fleisch, 2003). BPs may directly inhibit many MMPs *in vitro* (Teronen *et al*., 1999) and they preserve the joint architecture and decrease the inflammatory reaction in many types of experimental arthritis (Fleisch *et al*., 2002). However, in RA the anti-inflammatory properties of BPs and their potential efficacy in ameliorating structural damage remain to be elucidated.

3. REWIEV OF LITERATURE

3.1. Bone

3.1.1. General

Bone is a specialized form of connective tissue that regulates its mass and architecture to meet two critical, competitive and dynamically changing functions, structural and metabolic. Like other connective tissues bone consists of cells and extracellular matrix. The feature that distinguishes bone from other connective tissues is the unique ability of the bone matrix to become calcified. This produces an extremely hard tissue providing support and protection. Secondly, bone serves as a mineral reservoir and has an important role in the homeostatic regulation of blood calcium levels. The balance between structural and metabolic functions is achieved via complex and tightly regulated processes of formation and resorption of bone tissue.

3.1.2. Cellular basis of bone remodeling

The four cell types of bone tissue are osteoprogenitor cell (preosteoblast), osteoblast, osteocyte and osteoclast. With the exception of osteoclast, these cells can be regarded as differentiated forms of the same local mesenchymal stem cell. A mature osteblast is derived from preosteoblast and has histological characteristics that reflect its high cellular activity. It secretes both the bone collagen and the ground substance that constitute the initial unmineralized bone or *osteoid*. Osteoblast is also responsible for the calcification of the matrix. During the process of bone formation, the osteoblast may be engulfed in calcifying osteoid matrix produced by itself, thereby becoming the osteocyte. The osteocytes are connected to each other via cytoplasmic extensions that pass through a network of small canals known as *canaliculi*. The exchange of nutrients, mineral, and chemical and physical stimuli through this network is essential for the homeostatic control of skeleton. Monocyte-macrophage lineage derived osteoclasts are large multinucleated cells responsible for bone resorption (Rubin and Rubin, 2001).

3.1.3. Composition of bone matrix

3.1.3.1. Bone mineral

Bone is composed of ~70 % mineral and 30 % organic matter. The mineral, primarily in the form of hydroxyapatite [$Ca_{10}(PO4)_6(OH)_2$] crystals, is embedded in and aligned with the collagen fibrils, which together with noncollagenous proteins play an important role in crystal formation (Seibel *et al*., 1995).

3.1.3.2. Type I collagen

Type I collagen is the predominant organic component of bone, accounting approximately 85–90 % of unmineralized matrix. This protein is also the most abundant collagen in soft tissues e.g. skin and tendon. The bone collagen fibers are highly insoluble because of their many covalent intra- and intermolecular cross-links, the type and pattern of which differ from those in soft connective tissue collagen (Eyre *et al*., 1988). Type I collagen is com-

posed of two identical α1(I) chains and a structurally very similar but still genetically different α2(I) chain, which are wrapped around each other into a triple helix. Each triple helical collagen monomer, approximately 300 nm long, is aligned parallel to the next in a quarter-stagger array, which produces a collagen fibril. Within the collagen fibrils, gaps called hole zones exist between the end of the molecule and the beginning of the next. It is thought that noncollagenous proteins reside in these spaces, attract calcium and initiate the mineralization process (Rubin and Rubin, 2001).

3.1.3.3. Noncollagenous proteins

Although 90 % of the unmineralized matrix is collagen, the noncollagenous components that constitute the remaining 10 % are important in providing bone with some of its physical and chemical properties. The proteoglycan of bone is of lower molecular weight and is more compact than that in cartilage. Noncollagenous proteins include a calcium-binding, γ-carboxyglutamic acid-containing protein, osteocalcin and osteonectin, which is highly phosphorylated glycoprotein that binds collagen and calcium. Osteopontin and bone sialoprotein are highly acidic, have a high affinity for calcium and have binding sites for integrin receptors. All these proteins are probably important in regulating mineralization, and their distribution may account for the delay between matrix deposition and mineralization. These proteins may also be involved in bone resorption. Osteocalcin is chemotactic for osteoclasts and their precursors and osteopontin, as well as other proteins, may be involved in the adhesion of osteoclasts to mineralized matrix (Raisz, 2001).

3.1.4. Bone resorbing cells

The major and possibly the sole bone resorbing cell is the osteoclast. Osteoclasts are localized on endosteal bone surfaces, in Haversian systems and occasionally on periosteal surfaces. They are not commonly seen on resting bone surfaces but are found frequently at actively remodeling bones such as the metaphyses of growing bones or in pathological bone, such as adjacent to collections of tumor cells (Mundy, 1993).

The osteoclast is a polykaryocyte formed by fusion of mononuclear cells derived from hematopoietic bone marrow. The process of osteoclast differentiation requires the presence of receptor activator of nuclear factor kappa B ligand (RANKL) and the permissive factor macrophage colony stimulating factor (M-CSF) secreted by local osteoblasts and other stromal cells (Lacey *et al*., 1998). Together, RANKL and M-CSF induce expression of genes that typify the osteoclast lineage, including those encoding tartrate-resistant acidic phosphatase (TRAP), cathepsin K, calcitonin receptor and vitronectin receptor ($\alpha_v\beta_3$) (Lee *et al*., 1995; Faust *et al*., 1999).

Once activated the osteoclast attaches itself to the bone surface with the help of surface integrin αvβ3 receptor and forms a "seal" (clear zone) that demarcates the limits of the bone area being resorbed (Ross *et al*., 1993). Towards the center of the cell the membrane becomes deeply folded, creating a characteristic ruffled border. Via the ruffled border hydroclorid acid is secreted by the H^+ATPase to decalcify the bone, followed by the release of cathepsins which degrade bone matrix proteins. Degradation products (collagen fragments, solubilized calcium and phosphate) are internalized, transported across the cell and released at the basolateral domain (Salo *et al*., 1997). Once bone has been resorbed, the osteoclast disengages, leaving a resorption pit that is subsequently filled with new bone by osteoblasts (Väänänen and Horton, 1995).

3.1.5. Regulation of bone remodeling

Bone remodeling is a tightly coupled process regulating bone structure and function during adult life, with the key participants being the osteoclast and the osteoblast. In the normal adult skeleton bone formation occurs only where bone resorption has previously occurred. In young, healthy adult bone formation equals bone resorption, so that there is no net bone loss. However, with aging and in some diseases bone resorption exceeds bone formation resulting in generalized osteopenia osteoporosis and/or localized bone loss. Bone metabolism is in addition to physical forces and direct cell-to-cell contacts regulated by polypeptide, steroid and thyroid hormones as well as by local growth factors that play direct and important roles in bone remodeling (Canalis, 2003).

Parathyroid hormone (PTH) and 1,25 hydroxyvitamin D are both potent stimulators of osteoclastic bone resorption. This effect, however, is not direct, but requires the presence of osteoblasts or osteoblast-derived factors. Consistent with the tight coupling of the catabolic and anabolic phases of bone remodeling, the two major bone resorbing hormones (PTH and 1,25 hydroxyvitamin D) are also able to stimulate bone formation. This anabolic effect is mediated at least in part by the local insulin-like growth factor (IGF) I (Miyakoshi *et al*, 2001).

Calcitonin is a polypeptide hormone that is an inhibitor of osteoclastic bone resorption. The response to calcitonin is transitory, however, as osteoclasts escape from its inhibitory effects following continued exposure (Mundy, 1993).

Polypeptide growth factors, such as transforming growth factor (TFG)-β and IGF are secretory osteoblast products that can stimulate osteoblast cell growth in an autocrine or paracrine fashion. The active TFG-β and IGFs released from bone matrix via bone resorption could be instrumental in suppressing further bone degradation and initiating the bone forming phase of the remodeling cycle. Glucocorticoids inhibit skeletal IGF I synthesis, which may in part explain the inhibitory effect of glucocorticoids on bone formation (Canalis and Delany, 2002).

The recent identification and characterization of RANKL, an essential cytokine for various osteoclast functions, its receptor, receptor activator of nuclear factor kappa-B (RANK), and its decoy receptor, osteoprotegerin (OPG), have created new molecular and cellular concepts of osteoclast biology, bone resorption and homeostasis (Teitelbaum, 2000).

RANKL produced by osteoblasts, fibroblasts and activated T-lymphocytes is an essential factor for osteoclast formation, its fusion, activation and survival, thus resulting in bone resorption and bone loss (Figure 1). RANKL activates its specific receptor RANK located on preosteoclast, osteoclast and dendritic cell. RANK signal transduction involves many tumor necrosis factor receptor-associated factors including nuclear factor kappa-B (NF-κB) and activating protein-1 (AP-1), which consists of c-fos and c-jun (Boyle *et al*., 2003). The effects of RANKL are inhibited by OPG which acts as a soluble neutralizing receptor. RANKL and OPG act as key effector molecules onto which growth factors, cytokines, and peptide and steroid hormones known to effect bone metabolism converge (Hofbauer and Heudfelder, 2001).
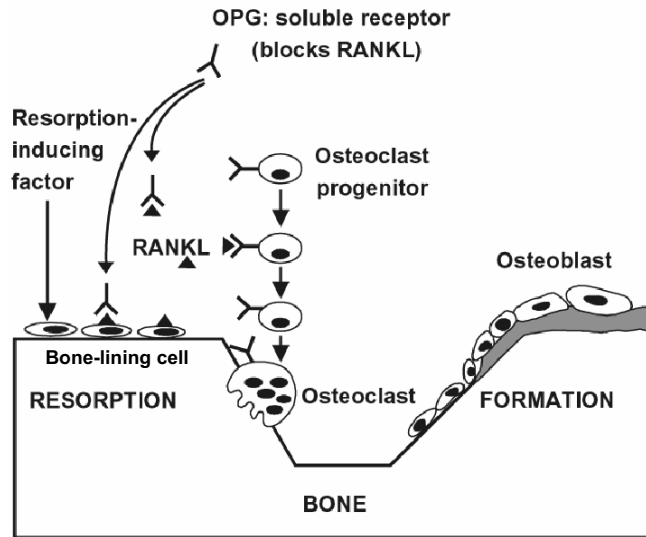
Figure 1. Osteoclast differentiation and activity is regulated by RANKL, RANK and OPG. OPG is a soluble decoy receptor that inhibits osteoclast differentiation and activation by binding to the cell surface RANKL and soluble RANKL, which are required for osteoclastogenesis.

S.R. Glodring. Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. Rheumatology 2003; 42(Suppl.2): ii11- ii16, by permission of Oxford University press.

3.2. Rheumatoid arthritis

3.2.1. General

Rheumatoid arthritis (RA) is a symmetric polyarticular arthritis that primarily affects the small diarthroidal joints of the hands and feet. RA is the most common inflammatory arthritis affecting about 1 % of the population worldwide and is a major cause of disability. Although the etiology of RA remains a mystery, a variety of studies, including twin concordance data, suggest that both environmental and genetic factors are responsible.

In the area of pathogenesis substantial progress has been made in recent years: The role of chemokines, adhesion molecules, cytokines, growth factors, and MMPs has been carefully defined. These products attract and activate immigrant cells in the peripheral blood and resident cells in tissues. The proteases can lead to behavior similar to that of localized tumor, resulting in invasion and destruction of articular cartilage, subchondral bone, tendons, and ligaments (Firestein, 2001).

3.2.2. Synovial abnormality in RA

The synovium is normally a relatively acellular structure with a delicate intimal lining. In RA, CD4^{+} T cells, B cells, plasma cells and macrophages infiltrate the synovium and sometimes organize into discrete lymphoid aggregates with germinal centers. Two major cell types are found in the lining: a macrophage-like cell or type A synoviocyte and fibroblast-like cell or type B synoviocyte. The numbers of type A and B synoviocytes are rela-

tively equal in normal synovium. There is an absolute increase in the numbers of both cell types in RA, although the percentage increase in macrophage-like cells is greater (Mulherin *et al.*, 1996).

Overall, within the synovium, T cells predominate over B cells. T cells constitute ≥ 50 % of cells in most RA synovia, whereas only ≤ 5 % of cells are B lymphocytes. However, only small amounts of T cell cytokines, e.g., interleukin (IL) -2 and interferon-γ, are found in inflamed joint, whereas cytokines and effectors produced by macrophages and fibroblasts abound. This finding has led to a concept that the chronic inflammatory process in RA might achieve a certain degree of autonomy that permits inflammation to persist after a T cell response has already been down-regulated (Firestein, 2001).

Synovial macrophages are believed to play a crucial role in the perpetuation phase of RA and their numbers correlate with articular destruction (Mulherin *et al.,* 1996). They release numerous cytokines, principally tumor necrosis factor (TNF) -α and IL -1β, which stimulate synovial fibroblast proliferation and secretion of IL-6, M-CSF and chemokines as well as effector molecules like MMPs and prostaglandins. The important role of innate immunity in RA is supported by clinical improvement observed after specific inhibition of macrophage-derived cytokines such as TNF-α and IL –1. However, continuous anti-cytokine treatment is required for long-term control and the disease flares when therapy is discontinued. Thus, a relatively simple view of autonomous cytokine networks cannot explain perpetuation of RA. More likely, the chronic synovial inflammation is dependent on components of the adaptive immune system as a driving force (Firestein, 2003). Recent observation that depletion of B cells in RA patients results in a significant therapeutic effect provides evidence for a pathogenic role for B cells in RA (Edwards *et al.*, 2004).

3.2.3. Cartilage destruction

Unlike bone, which is constantly remodeled throughout life, cartilage turnover (remodeling) is relatively limited and this tissue has restricted capacity to repair its matrix once it is damaged. In RA cartilage is destroyed by both enzymatic and mechanical processes. Early in synovitis, proteoglycans are depleted from cartilage, which looses its ability to rebound from a deforming load and thereby becomes susceptible to mechanical fragmentation and fibrillation and eventually looses its functional integrity concurrent with the degradation of fibrillar type II cartilage collagen by collagenolytic enzymes (Firestein, 2001).

3.2.4. Pathogenesis of bone destruction in RA

The presence of juxta-articular osteoporosis, focal bone erosions at joint margins and bone cysts in the subchondral bone has been considered as the radiographic hallmarks of RA (Goldring and Gravallese, 2002). There is mounting evidence that osteoclast plays a pivotal role in RA associated local and generalized bone loss. Histologic sections of rheumatoid joints obtained from patients at the time of joint replacement surgery demonstrate multinucleated cells with osteoclastic phenotype along the surface of resorption lacunae in subchondral bone (Hummel *et al.*, 1998; Gravallese *et al.*, 1998). The phenotypic markers that are associated with the fully differentiated osteoclast include cathepsin K, TRAP and calcitonin receptor. Expression of calcitonin receptor coincides with the terminal differentiation of the osteoclast into a fully competent bone resorbing cell, which makes it

a useful marker for the identification of the mature functional osteoclasts and helps to distinguish this cell from its monocyte/macrophage-lineage precursors (Gravallese *et al.*, 1998).

The origin of the osteoclast like cells found at the sites of focal bone erosions at the pannus-bone interface remains unclear. Rheumatoid synovium is rich in macrophages. The cells share the same origin as osteoclasts and can be induced *in vitro* to differentiate into mature active osteoclasts fully capable of resorbing bone (Fujikawa *et al.*,1996). It is conceivable that the multinucleated cells at the pannus-bone junction are derived from synovial macrophages in rheumatoid joints, but this has not been proven.

Several lines of evidence indicate a role for RANKL in the pathogenesis of focal bone loss in RA (Gravallese *et al.*, 2000; Horwood *et al.*, 1999; Kotake *et al.*, 2001). RANKL is expressed in osteoblasts, synovial fibroblasts and T-cells, all of which have been shown to have the capacity to induce osteoclastogenesis *in vitro* (Horwood *et al.*, 1999; Kotake *et al.*, 2001). IL-1 and TNF-α have dual effects on osteoclastogenesis. Both factors up-regulate RANKL expression in bone lining and bone marrow stromal cells. In addition, TNF-α and RANKL act in synergy to enhance osteoclast differentiation, whereas IL-1 acts primarily to directly activate osteoclasts and delays osteoclast apoptosis (Romas et al., 2002). Both IL-1 and TNF-α contribute further to bone loss in RA by impairing bone formation via induction of osteoblast apoptosis (Tsuboi *et al.*, 1999). Consequently the beneficial effects of targeting IL-1 and TNF-α for retarding the progression of bone erosions in RA may not only reflect the suppression of synovial inflammation, but could relate to the ability of these therapies to interfere directly with osteoclastogenesis, as well. Recent observations in the rat adjuvant arthritis model demonstrating blockade of focal bone loss in animals treated with OPG provide further evidence that RANKL plays a critical role in the pathogenesis of the osteoclastic bone resorption in inflammatory arthritis, and that the osteoclast is the principal cell type responsible for the development of erosions at the pannus-bone interface (Kong *et al.*, 1999).

3.2.5. Evaluation of radiographic joint damage

Joint damage visualized by radiographs is considered to be the hallmark of RA. Although the course of RA varies among individual patients, there are broadly identifiable patterns and these can be defined using plain X-rays (Plant *et al.*,1998). With increasing duration of disease there is an increasing relationship between joint damage visualized on plain films and several aspects of outcome, such as functional status and work disability (Scott *et al.*, 2000). Therefore it is important to assess the progression of structural damage in individual patients in clinical practice and if, necessary, to adjust therapy accordingly. The extent of damage caused by RA varies in different joints. However, damage to the hands and feet provides a good indication of overall changes in time. Therefore, the use of the hands and feet as surrogate measure for overall joint damage is favored (van der Heijde *et al.*, 1999a).

To be able to use radiographs as an outcome measure, valid methods are needed to score progression of radiologic changes in RA. The earliest systems developed by Steinbocker *et al.* (1949) and Kellgren and Bier (1956) gave global assessments of the extent of damage, which allowed individual patients to be classified into groups. These groups are useful in epidemiological studies but have limited relevance when assessing the progres-

sion of damage in trials. Subsequent systems assessed changes in individual joints. The dominant methods are the Sharp score (Sharp *et al.*, 1971), the Larsen score (Larsen *et al.*, 1977), and their many variants. The key modification is the van der Heijde modificatied Sharp score, which involves assessing 16 joint in each hand and wrist and each foot on ordinal scales for erosion (0–5 in hands and 1–10 in feet) or joint space narrowing (0–4) (van der Heijde, 1999b). The Larsen method is based on a set of standard films. It differentiates six stages from 0 (normal) to 5, reflecting gradual, progressive deterioration, and provides an overall measure of joint damage. Of these two scoring systems the Sharp/van der Heijde method was reported to be more sensitive to detect change (Bruynesteyn *et al.*, 2002).

X-rays can be scored in random order, in pairs without knowledge of the chronological sequence, or in known sequence. Scoring in chronological order may introduce a bias because it is expected that damage will progress over time. However, chronological order is more sensitive to change than the other approaches, and this difference is particularly pronounced with longer follow up (van der Heijde *et al.*, 1999c).

3.3. Joint destruction and proteinases

3.3.1. General

Degradation of extracellular matrix components is an integral feature of normal growth and development, and biologic processes such as reproduction, bone remodeling and wound healing. Under physiologic conditions proteolytic degradation is transient, local, and controlled by endogenous inhibitors. In many pathologic situations, excessive extracellular matrix degadation by proteinases causes tissue destruction. Joint destruction in RA is attributed mainly to elevated proteinase activities without sufficient endogenous inhibitors (Okada, 2001). The four main classes of proteinases are classified according to the chemical group that participates in the hydrolysis of peptide bonds. Cysteine and aspartate proteinases are predominantly active at acid pH and act intracellularly; the serine and metalloproteinases, active at neutral pH, act extracellularly. The proteinases produced by chondrocytes play a major role in osteoarthritis (OA), while in rheumatoid joint proteinases produced by chondrocytes, synovial cells and inflammatory cells all contribute to matrix loss (Catterall and Cawston, 2003).

3.3.2. Metalloproteinases

Based on the substrate specificity the family of MMPs is subdivided to subgroups such as collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3 and -10) and membrane-type MMPs (MMP-14, -15,-16, -17). MMPs share a structurally similar domain structure, in particular the zinc dependent catalytic domain and the activation peptide (propeptide) thought to be responsible for the latency of proMMP species (Konttinen *et al.*, 1999).

3.3.2.1. Collagenases

Collagenases are characterized by their ability to degrade triple helix of interstitial collagen types I, II and III at a specific single site following a glycine residue, located about three fourths distance from the N terminus. This cleavage generates fragments approximately three fourths and one fourth of the size of the original molecule. Fibroblast type collagenase, later renamed MMP-1, was the first MMP detected in the rheumatoid synovial membrane (Evanson *et al.*, 1967). Neutrophil collagenase or MMP-8 is synthesized during the differentiation of neutrophils and stored in the secondary or specific granules until the neutrophil is activated to exocytosis (Okada, 2001). A newly described collagenase, MMP-13, is highly expressed in chondrocytes in both OA and RA cartilage. In addition, MMP-13 is produced in RA synovium in amount considerably higher than OA (Lindy *et al.*, 1997). The importance of MMP-13 in cartilage destruction is suggested by its substrate specificity favoring degradation of type II collagen over type I and III collagens (Knäuper *et al.*,1996).

3.3.2.2. Gelatinases

Among the MMPs MMP-2 (gelatinase A) and MMP-9 (gelatinase B) may be especially important in collagen degradation through digestion of denatured collagen (gelatin) generated by thermal denaturation of the 3/4 and 1/4 collagen fragments at body temperature after specific cleavage of the triple helical region of the fibrillar collagen molecules by collagenases (Okada, 2001). MMP-2 is expressed mainly by mesenchymal cells, i.e., rheumatoid synovial fibroblasts and chondrocytes, whereas MMP-9 is produced by neutrophils and various other hematopoietic cells, for instance, monocytes and macrophages (Opdenakker *et al.*, 2001). Gelatinases cleave type IV collagen, the main component of basement membrane (BM), and MMP-9 from macrophages and neutrophils is thought to play a key role in the migration of these cells in inflammatory diseases such as RA (Jovanovic *et al.*, 2000).

3.3.2.3. Stromelysins

The closely related stromelysins, MMP-3 and MMP-10, hydrolyze a number of ECM macromolecules including fibronectin, laminin, collagen IV and aggregan, the major proteoglycan species in cartilage. Stromelysins can activate other MMPs, thus playing an important role in the intermolecular activation cascade of MMPs (Okada, 2001).

3.3.3. Cysteine proteinases

ECM degrading cysteine proteinases include lysosomal cathepsins B, L, S and K. Cathepsins B and L digest the telopeptide regions of fibrillar collagen and aggregan at acidic pH. Cathepsin S has a similar spectrum of substrates within a broad range of pH. Cathepsin K is a collagenolytic cathepsin that effectively cleaves type I collagen at several sites of the triple helical region at pH values between 4.5 and 7.6. Cathepsin K also degrades gelatin and osteonectin (Okada, 2001). Cathepsin K is highly expressed in osteoclasts and its critical involvement in bone remodeling is supported by the finding that cathepsin K deficiency causes bone sclerosing disorder pycnodysostosis that on the molecular level is characterized by insufficient degradation of type I collagen during bone remodeling (Gelb *et al.*, 1996; Hou *et al.*,1999).

3.3.4. Regulation of proteinase activity

3.3.4.1.Regulation of metalloproteinase production

Although most MMPs are not expressed in normal cells under physiologic conditions, MMP-8 and MMP-9 are synthesized during the differentiation of neutrophils and stored in the granules of differentiated cells (Okada, 2001). The expression of MMPs by connective tissue cells is regulated by a number of cytokines (particularly IL-1 and TNF-α), growth factors, and hormones. Many of these factors are products of monocyte/macrophages and their production in inflammatory situations is a key step in the initiation tissue degradation (Reynolds, 1996).These regulatory signals work almost exclusively at the level of gene transcription and involve multiple mechanisms such as activation of AP-1 and NF-κB transcription factors (Liacini *et al*., 2002). MMP-2 is unique in that factors capable of enhancing the production of other MMPs are inactive, whereas TGF-β, suppressor for most MMPs, stimulates its production (Okada, 2001).

3.3.4.2. Activation mechanisms of the zymogens of MMPs

MMPs are synthesized as latent proenzymes. The latency of proMMPs is maintained by interaction of the cysteine sulfhydryl group in the pro-domain, with the zinc atom at the active site. This prevents the formation of a water-zinc complex that is required for the enzyme catalyzed reaction. The extracellular activation is initiated through the disruption of the cysteine-zinc interaction by treatment with nonproteolytic agents or proteinases. It is likely that *in vivo* activation proceeds most frequently by proteolysis. Proteinases initially cleave the pro-peptide so that the cysteine is no longer held in a tight apposition to the zinc atom. Autolysis ensues to produce the cleaved, permanently active form of enzyme. Many serine proteinases such as plasmin may play a major role in the activation of proMMP-3 and proMMP-10 *in vivo*. Activation of proMMP-1, -8 and -9 by active MMP-3 and MMP-10 may then be the next steps in this intermolecular activation cascade. ProMMP-9 may be activated by MMP-13 as well. ProMMP-2 which is resistant to extracellular activation by most endopeptidases can be activated by membrane-type MMPs, MMP-14, -15 and -16 on the cell membranes (Okada, 2001).

3.3.4.3. Endogenous proteinase inhibitors

Most of the proteinase inhibitory activity in serum is due to α_2-macroglobulin (α_2M). Because of its large molecular weight, it is not present in non-inflammatory synovial fluid. During synovial inflammation, α_2-macroglobulin penetrates into the joint cavity. Rheumatoid synovial fluid, for example, has about the same concentration of the inhibitor as plasma. Tissue inhibitors of metalloproteinases (TIMP), of which there are four to date, block the enzyme activity by binding to MMPs in a 1:1 molar ratio to form tight, noncovalent complexes (Okada, 2001). Normally, a tight balance exists between MMPs and their tissue inhibitors. However, in pathological situations, such as RA, a MMP/TIMP imbalance is present, which leads to an excess of activated MMPs, and ultimately, to structural damage in joints (Firestein, 2001).

3.3.5. Cartilage destruction by proteinases in RA

Although several proteinases are known to attack and degrade the proteoglycan component of cartilage, degradation of fibrillar collagen is thought to be mediated only by the

collagenase enzymes. Recent evidence suggests, however, that cathepsin K expressed in synovial fibroblasts in RA may contribute to this event as well (Hou W-S *et al*., 2001). Probably all the three collagenases are involved. Chondrocytes, stimulated by the cytokines derived from inflamed synovium, produce MMP-13, which of the three collagenases most effectively cleaves type II collagen (Knäuper *et al*.,1996). Immunolocalization of MMP-1 and phagosytosis of collagen fibrils by pannus cells at sites of pannus-cartilage junction suggests a role for MMP-1 in cartilage destruction (Okada, 2001). Rheumatoid synovial effusions typically contain large numbers of neutrophils (PMN). MMP-8 degranulated from PMNs interacting with immunoglobulins adherent to cartilage and activated by hypochlorous acid released as part of the PMN activation, is likely to contribute to cartilage destruction, because collagenase released contiguous to the cartilage matrix might escape the circulating protease inhibitors (Chatham *et al*.,1990).

3.3.6. Proteinases in bone resorption

Pannus-like granulation tissue invades the cartilage and subchondral bone at the bare zone, and bone resorption occurs through the action of osteoclasts. Osteoclastic bone matrix degradation is carried out in the subosteoclastic compartments, which have acidic (pH 4-5) and hypercalcemic conditions. After solubilization of the mineral phase, the organic matrix is degraded. Both *in vitro* and *in vivo* studies suggest that this process is mainly carried out by lysosomal cysteine proteinases and MMPs (Everts *et al*., 1992; Everts *et al*., 1998). Data that cathepsin K is the major proteinase in the degradation of bone matrix in the resorption lacunae are now very convincing. The enzyme has telopeptidase activity like other cysteine proteinases, and it shares with bacterial proteinases the ability to cleave collagen at multiple sites. Thus, cathepsin K can both depolymerize collagen fibers and cleave triple helices (Garnero *et al*., 1998). Moreover, the pH for optimal activity of cathepsin K is within the range of pH values measured in the resorption zone of osteoclasts.

MMP-9 is the best established MMP in osteoclast and it is highly expressed by these cells. It has telopeptidase activity against soluble and insoluble type I collagen and strong gelatinolytic activity. ProMMP-9 is activated by acid exposure followed by neutralization, and once activated, it is proteolytically active under acidic and hypercalcemic conditions (Okada, 2001). MMP-9 has strong proteolytic activity against type IV collagen and it is likely that osteoclasts and/or preosteoclasts require this enzyme for migration (Inui *et al*., 1999).

A line of evidence suggests that also MMPs of non-osteoclastic origin contribute to the collagenolysis in bone (Garnero *et al*., 2003). Particularly MMP-13, produced by osteoblast-lineage cells, may be involved in degradation of organic components of bone matrix, acting in concert with cathepsin K and MMP-9 produced by osteoclasts (Nakamura *et al*., 2004). Moreover, the relative importance of MMPs and cathepsin K in bone resorption may depend on the given physiological situation. Thus, MMPs are more important for resorption of calvarie compared with long bones (Delaissé *et al*., 2000). Cathepsin K is the prevailing collagenolytic enzyme in normal physiological bone remodeling, whereas MMPs become more important in osteolysis related to pathologic situations, including metastatic bone disease and multiple myeloma (Garnero *et al*., 2003).

3.3.7. MMPs as regulators of inflammatory response

In order to clarify the role of gelatinases in arthritis, Itoh *et al*. (2002) investigated the development of antibody-induced arthritis in MMP-2 or MMP-9 knockout mice. Surprisingly, the MMP-2 knockout mice exhibited a more severe clinical and histologic arthritis than wild-type mice, whereas MMP-9 knockout mice displayed milder artrhitis. These results indicate a suppressive role of MMP-2 and an enhancing role of MMP-9 in the development of inflammatory joint disease. As these enzymes have very similar substrate specificities for matrix proteins (Woessner, 1991), their opposite roles may be caused by differences in non-matrix substrates (Itoh *et al*., 2002).

For example, MMP-2 cleaves and inactivates monocyte chemoattractant protein-3 (MCP-3) (McQuibban *et al*., 2000) and can contribute to the dissipation of proinflammatory activities. MCP-3 is not cleaved by MMP-9, whereas the major neutrophilic chemoat-tractant IL-8 is amino-terminally truncated and potentiated into a chemokine with at least tenfold greater activity (van der Steen *et al*., 2000). Moreover, MMP-9 very effectively processes IL-1β precursor, yielding stable bioactive products (Schönbeck *et al*., 1998).

Taken together, MMPs can be seen both as effectors and regulators of inflammatory response and they can act both as enhancers and suppressors in the progression of RA. This points out the difficulty of using non-selective MMP inhibitors for the treatment of RA. The development of selective inhibitors, especially highly specific for MMP-9, would be essential.

Recent findings suggest an important role for neutrophil-derived MMPs, MMP-8 and MMP-9, in the induction of immunopathology of various autoimmune diseases (Opde-nakker *et al*., 2001; van den Steen *et al*, 2002). In the joint, activated neutrophils degran-ulate MMP-8 and MMP-9, but produce neither MMP-2 nor TIMPs. MMP-8 cleaves car-tilage type II collagen at a single site and generates the typical 3/4 and 1/4 fragments which leads to unwinding of the triple-helix. Further cleavage by MMP-9 and the resulting release of auto-immunodominant epitopes could contribute to the establishment and per-petuation of RA (van den Steen *et al*., 2002).

3.4. Biochemical markers of bone metabolism

3.4.1. Markers of bone formation

Metabolism of bone mineral being difficult to assess reliably, analysis of type I collagen has been developed for quantification of metabolic turnover in skeleton. During the syn-thesis of type I collagen, by osteoblasts in bone and by fibroblasts in other connective tissues, the aminoterminal (PINP) and carboxyterminal (PICP) extension propeptides are cleaved off (Risteli and Risteli, 1999). PINP, in particular, has been found to be a sensitive and dynamic marker of bone formation (Scariano *et al*., 1997; Melkko *et al*., 1996). In women with nonmetastatic breast cancer treated with clodronate, changes in PINP levels significantly predicted changes in BMD (Saarto *et al*., 1998). Furthermore, the percent-age decrease in PINP significantly predicted the decreased risk of vertebral fractures observed in subjects treated with raloxifene (Reginster *et al*., 2004). PINP is elevated in patients with RA compared to controls, but PINP levels do not correlate with disease activity (Cortet *et al*, 1998; Hakala *et al*., 1995).

Osteocalcin, a major noncollagenous matrix protein of bone, is produced exclusively by osteoblasts. It is a marker of bone formation that correlates with histomorphometric bone measurements (Delmas *et al.*, 1985). In most conditions, bone resorption and formation are tightly coupled, and therefore osteocalcin, PINP and PICP levels reflect bone turnover.

3.4.2. Degradation products of type I collagen

The most promising clinical markers of bone resorption are based on collagen degradation products, in particular cross-linking amino acids and cross-linked telopeptides. Pyridino-lines are trifunctional cross-links present in collagens of all major connective tissue. They covalently link collagen molecules between two telopeptides and a triple helical sequence at two intermolecular sites, N-telopeptide – to – helix and C-telopeptide – to – helix (Apone *et al.*, 1997). The newly developed immunoassays quantify, in serum and/or in urine, different degradation products originating from both the N- and C-telopeptides (Hanson *et al.*, 1992; Risteli *et al.*, 1993, Bonde *et al.*, 1994).

3.4.2.1. NTx assay

The cross-linked $\alpha2(I)$ N-telopeptide in urine and serum, referred to as NTx, has proven to be a sensitive marker of bone resorption measured by microtiter plate ELISA using monoclonal antibody, mAb 1H11 (Hanson *et al.*, 1992; Clemens *et al.*, 1997). MAb 1H11 binds to an $\alpha2(I)$ N-telopeptide epitope, but only when this domain is cross-linked through its lysine- residue and cleaved proteolytically to the eight-amino acid sequence shown in Figure 2 (Hanson and Eyre, 1996). *In vitro*, recombinant human cathepsin K can release

Figure 2. Structure and molecular origin of the antigenic epitope in the aminoterminal (NTx) telopeptide region of human type I collagen molecule. The required feature of the epitope recognized by monoclonal antibody 1H11 is the a2(I) N-telopeptide sequence, JUDGKGVG, where K is embodied in trivalent cross-links.

Reprinted from BONE, Vol 26, Atley LM, Mort JS, Lalumiere M, Eyre DR, Proteolysis of human bone collagen by cathepsin K: characterization of the cleavage sites generating by cross-linked N-telopeptide neoepitope, Pages 241-7, Copyright 2000, with permission from Elsevier.
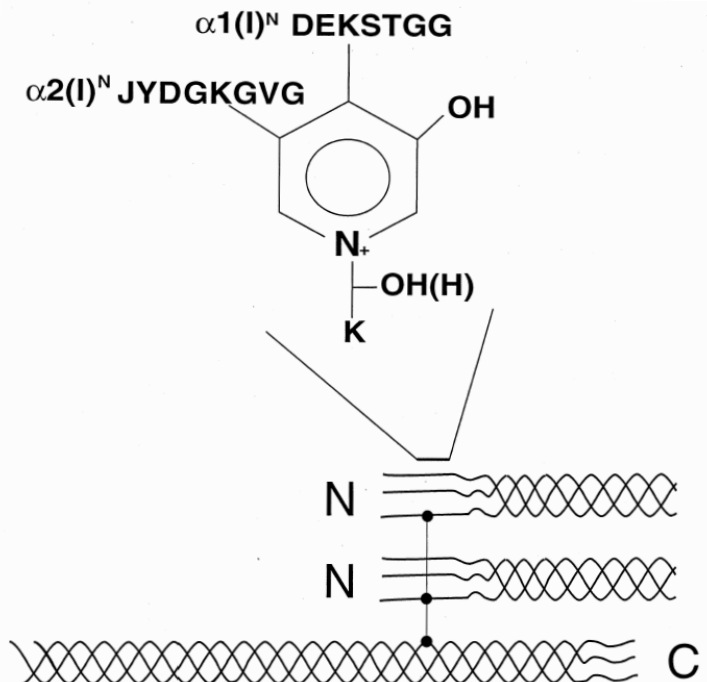
all the latent NTx epitope from either soluble, denatured bone collagen or from insoluble, demineralized bone matrix (Atley *et al*, 2000). Given the restricted distribution of cathepsin K to osteoclasts, these findings provide a molecular explanation for the specificity and sensitivity of NTx as a systemic marker of osteoclastic bone resorption (Atley *et al*., 2000). Urinary and serum levels of NTx are elevated when bone resorption is accelerated, for example, in women after menopause and lowered by therapies known to inhibit bone resorption (Garnero *et al*., 1994; Clemens *et al*, 1997; Scariano *et al*., 1998). Increased urinary excretion of NTx has been reported in active RA (Al-Awadhi *et al*., 1999), but to date there are no published studies applying the newly developed enzyme linked immunosorbent assay (ELISA) for serum NTx (Clemens *et al*., 1997) in arthritic conditions.

3.4.2.2. CTx and ICTP assays

Two different circulating C-terminal fragments of type I collagen, known as ICTP and carboxyterminal cross-linked peptide of type I collagen (CTx) have been used as markers of bone degradation *in vivo*. The original CrossLaps assay was developed for an eight amino acid long synthetic peptide involving the cross-link site of the carboxyterminal telopeptide of the $\alpha 1$(I) chain as an unmodified lysine (Bonde *et al*., 1994). Serum and urinary CTx levels are markedly increased in postmenopausal women compared with premenopausal controls, and their values decrease markedly and shortly after initiation of antiresorptive treatment, including bisphosphonates, in postmenopausal women with osteoporosis (Garnero *et al*., 1994). Test tube assays show the ability of cathepsin K, but not MMPs, to generate CTx from insoluble bone collagen. However, bone resorption-related MMPs can contribute to CTx immunoreactivity in native tissue by further degrading some of the larger type I collagen fragments generated by cathepsin K (Garnero *et al*., 1998; Garnero *et al*., 2003).

The ICTP antigen is a trivalently cross-linked structure that was originally isolated from human femoral bone after digestion with trypsin or bacterial collagenase and shown to contain the carboxyterminal telopeptides of two $\alpha 1$(I) chains and material from the helical part of a third chain (Risteli *et al*.,1993). MMPs -2, -9, -13 and -14 are capable of releasing ICTP from bone collagen (Garnero *et al*., 2003; Parikka *et al*., 2001), whereas cathepsin K destroys the ICTP epitope (Sassi *et al*., 2000). Thus, among known collagenolytic proteinases relevant in bone resorption, only MMPs can generate ICTP. ICTP will, however, underestimate MMP driven collagenolysis in all situations where ICTP is exposed to active cathepsin K (Garnero *et al*., 2003).

These observations provide a mechanistic explanation, why ICTP is relatively insensitive to changes in osteoclast-mediated normal turnover of bone collagen. However, pathologic increases in bone degradation, such as those occurring in myeloma (Elomaa *et al*., 1992) or metastatic bone disease (Blomqvist *et al*., 1996) are well detected by this assay suggesting that other pathways, possibly resembling the extracellular degradative pathway of collagen, must also exist (Risteli and Risteli, 1999). Serum ICTP is elevated in RA patients and correlates with disease activity and radiologic damage score (Hakala *et al*.,1993).

In pycnodysostotic patients lacking cathepsin K, serum concentrations of ICTP are increased concomitantly with decreased urinary excretion of NTx and CTx (Nishi *et al.,* 1999). Plausible explanation for these findings is that MMPs from periosteoclastic cells compensate for inactive cathepsin K by degrading collagen that was demineralized by the

osteoclasts, and thereby generate ICTP (Garnero *et al.*, 2003). Absence of cathepsin K mediated cleavage of ICTP fragments, however, could also contribute to the increase in serum ICTP (Parikka *et al.*, 2001).

3.4.3. Osteprotegerin

OPG is soluble secreted receptor for RANKL that prevents it from binding to and activating RANKL on the ostaclast surface. OPG is produced by a variety of tissues including the cardiovascular system, lung, kidneys and bones. While abnormalities in the RANKL/OPG system have been implicated in the pathogenesis of osteoporosis and other metabolic bone diseases, including RA (Hofbauer and Heufelder, 2001), clinical and preclinical data suggest that OPG could have application as a potent antiresorptive drug (Bekker *et al.*, 2001; Kong *et al.*, 1999). The role of serum OPG measurement, however, as a biochemical marker for disease activity assessment and drug monitoring has not yet been fully elucidated (Hofbauer and Schoppet, 2001).
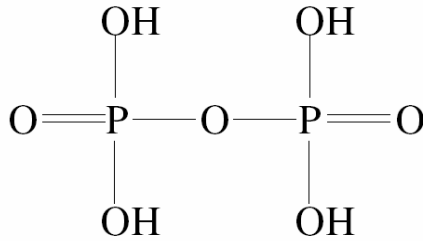
3.5. Bisphosphonates

3.5.1. General

In the early 1960s Fleisch and Bisaz (1962) showed that inorganic pyrophosphate, a known by-product of many biosynthetic reactions in the body, was present in serum and urine and prevented ectopic calcification by binding to newly forming crystals of hydroxyapatite. Due to its rapid hyrdolysis, however, pyrophosphate found therapeutic use only in scintigraphy and in toothpaste, added to prevent dental calculus. This prompted the search for analogs, which would display similar physicochemical properties, but resist enzymatic hydrolysis and metabolism. The bisphosphonates (BPs) fulfilled these prerequisites (Fleisch *et al.*, 2002). The finding that BPs inhibited the dissolution of hydroxyapatite crystals (Fleisch *et al.*, 1969) led to studies to determine whether they might also inhibit bone resorption. Since then, BPs have become established as an effective and safe treatment of bone loss, especially in osteoporosis.

3.5.2. Structure-activity relationship for bone resorption

Bisphosphonates differ from pyrophosphate in that a carbon rather than oxygen atom bridges the two phosphate residues (Figure 3), which renders BPs stable and able to withstand incubation in acids or with hydrolytic enzymes The P-C-P structure of BPs is required for the chelation of Ca^{2+} ions by these compounds and hence for their tissue selective targeting to bone mineral. The affinity for calcium can be increased further if one of side chains (R^1) is a hydroxyl (-OH) group, because this allows the formation of a tridentate conformation that is able to bind Ca^{2+} more effectively (Russel and Rogers, 1999). With the exception of clodronate, all clinically used BPs to date contain – H or – OH in the R^1 position. Etidronate, which has a hydroxyl group at the R^1 position, was, however, found to be less potent than clodronate in inhibition of bone resorption both *in vitro* and *in vivo*. This discrepancy between antiresorptive potency and affinity for Ca^{2+} led to the suggestion that BPs may inhibit bone resorption by cellular effects on bone resorbing cells rather than by acting as crystal poisons that prevent hydroxyapatite crystal dissolution by a physiochemical mechanism (Russel and Rogers, 1999).

Pyrophosphate

$$O=\!\!\overset{\displaystyle OH}{\underset{\displaystyle OH}{P}}\!\!-O-\!\!\overset{\displaystyle OH}{\underset{\displaystyle OH}{P}}\!\!=O$$

Generic Bisphosphonate

$$O=\!\!\overset{\displaystyle OH}{\underset{\displaystyle OH}{P}}\!\!-\!\!\overset{\displaystyle R1}{\underset{\displaystyle R2}{C}}\!\!-\!\!\overset{\displaystyle OH}{\underset{\displaystyle OH}{P}}\!\!=O$$
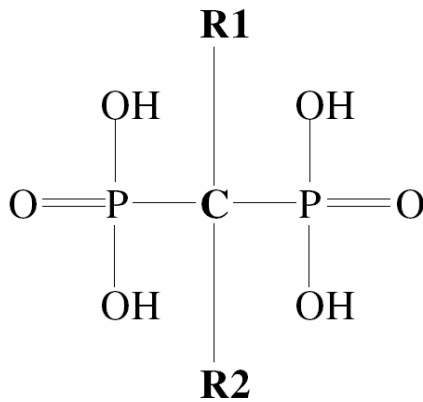
R1 enhances binding to hydroxyapatite
-C- enhances chemical stability
R2 determines anti-resorptive potency

Figure 3. Structure of pyrophosphate and generic bisphosphonate (Arthritis Res 5: 12-24, 2003).

Following the successful clinical use of clodronate and etidronate in the 1970s and 1980s, more potent antiresorptive BPs were developed, which had different R^2 side chains, but in which R^1 was unaltered. Extensive modifications of the side chain showed that basic primary nitrogen group attached to an alkyl chain, such as in pamidronate and alendronate (Table 1), produced 10 - 1000 - fold more potent antiresorptive agents. When the nitrogen atom was combined as a tertiary amine in the R^2 side chain, such as in ibandronate, the BPs were even more potent. However, the most potent BPs to date are those containing a tertiary nitrogen within a ring structure, including risedronate and zolendronate, which are up to 10.000 – fold more potent than etidronate *in vivo* in rodent models of bone resorption (Fleisch *et al*., 2002).

Table 1. Structure of the R^1 and R^2 sidechains (see Fig. 2) of some of the bisphospho-nates investigated in humans. The bisphosphonates are grouped according to their poten-cy for inhibiting bone resorption in rats.

Bisphosphonate	R^1	R^2
Potency x1		
Etidronate	OH	CH_3
Potency 10 x		
Clodronate	Cl	Cl
Tiludronate	H	CH_2-S-phenyl- Cl
Potency 100 x		
Pamidronate	OH	$CH_2 CH_2 NH_2$
Potency 100-1000 x		
Alendronate	OH	$(CH_2)_3 NH_2$
Potency 1000-10 000 x		
Ibandronate	OH	$CH_2 CH_2 N(CH_3)$ (pentyl)
Risedronate	OH	CH_2-3-pyridine
Potency >10 000 x		
Zoledronate	OH	CH_2-imidazole

3.5.3. Targeting of BPs in bone

The clinicil pharmacology of BPs is characterized by low intestinal absorption as a conse-quence of their poor lipophilicity, but highly selective localization to and retention in bone. The circulating levels of BPs are extremely low due to their affinity to bone mineral, suggesting that the circulating levels are not relevant to function. Their distribution in bones is not homogenous as BPs bind preferentially to bones which have high turnover rates. Following IV administration of [14]C- alendronate to rats the joint (mainly juxta-artic-ular trabecular bone) of the tibia and femur had two- to threefold higher drug concentra-tions than the middle portions (cortical bone) Using a light microscopy autoradiography technique, Sato *et al*. (1991) showed that only the exposed hydroxyapatite at the resorp-tion site is accessible to the circulating BPs, and this would partly explain the nonuniform distribution of BPs in bone. However, other factors, such as blood supply and ratio of bone surface to volume, may also contribute to this uneven distribution. The blood supply to trabecular bone is greater than to cortical bone, and its surface to volume ratio is approximately 4- fold compared to cortical bone both of which may lead to the higher drug concentrations observed in juxta-articular bone (Lin, 1996).

The ability of BPs to chelate Ca^{2+} is reduced at low pH due to the protonation of the phosphonate groups. Hence, in the acidic environment of the osteoclast resorption lacuna, BPs are released from bone surfaces, giving rise to locally high concentrations of BPs in solution or as calcium salt. For instance, it has been estimated that pharmacological doses

of alendronate that inhibit bone resorption *in vivo* could give rise to local concentrations as high a 1 mM alendronate in resorption space beneath an osteoclast (Sato *et al.*, 1991).

3.5.4. Mechanism of action

The tissue specific targeting of BPs to bone mineral, especially to sites of osteoclast activity, suggests that BPs inhibit bone resorption by direct effects on osteoclasts or other bone cells in the immediate microenvironment of osteoclasts. BPs affect osteoclast-mediated bone-resorption in a variety of ways, which include effects on osteoclast recruitment, differentiation and resorption activity. Cellular uptake of BPs leads to the loss of the ruffled border between the osteoclast and the bone surface, to the disruption of cytoskleleton and loss of function (Russel and Rogers, 1999).

Recent mechanistic studies show that bisphosphonates can be classified into at least two groups with different modes of action: those that are metabolized within the cell to form toxic analogs of ATP (e.g., clodronate, etidronate and tilundronate) and those that inhibit farnesyl diphosphate synthase (e.g., pamidronate, alendronate, ibandronate, risedronate and zolendronate).The properties that segregate BPs into these two classes appear to be a function of the moieties attached to the geminal carbon at R^2, which can vary in size and complexity. The prevailing determinant for mechanism of action of the BPs used in the clinics relates to the presence or absence of a nitrogen atom located three or five positions away from the geminal carbon of the P-C-P backbone in the in the R^2 group (Fleisch *et al.*, 2002).

The potent nitrogen containing BPs (N-BPs), such as alendronate and pamidronate, inhibit bone resorption by preventing protein prenylation in osteoclasts, owing to inhibition of farnesyl diphosphate synthase, an enzyme in the mevalonate pathway. The posttranscriptional modification with lipids (prenylation) of small GTPases such as Ras, Rho and Rac is essential for the correct function of these enzymes. These small GTPases control the osteoclast cytoskeletal arrangement, membrane ruffling, the trafficking of vesicles, and apoptosis. Therefore it is believed that inhibition of the prenylation of these small GTPases by N-BPs accounts for the majority, if not all, of the various effects on osteoclasts (Russel and Rogers, 1999).

The non-N-BPs that have a structure similar to pyrophosphate (e.g. clodronate and etidronate) do not inhibit protein prenylation but can be incorporated into nonhydrolysable analogues of ATP, via the cytoplasmic amino-acyl tRNA enzymes. ATP analogues accumulate within the cytoplasm, where they interfere with numerous biological processes, eventually causing both osteoclast and macrophage apoptosis (Frith *et al.*,2001).

3.5.5. Bisphosphonates as MMP inhibitors

BPs have been shown to inhibit the catalytic activities of several genetically distinct, but structurally related MMP family members *in vitro*. The IC_{50}s range from 50 to 150 μM (Teronen *et al.*, 1999). Because MMPs are zinc-dependant endopeptidases, it has been suggested that the bone hook of BPs (the P-C-P structure) inhibits the proteolytic activity of MMPs through chelation of divalent cations. In line with this postulation, all of the BPs used in clinics are equipotent in inhibiting MMP proteolytic activity despite the structural differences in their bioactive moiety (i.e. the R^2 chain) (Boissier *et al.*, 2000).

Tilundronate had no effect on either messenger ribonucleic acid (mRNA) or protein levels for MMP-1 and MMP-3 in periodontal ligament cells (Nakaya *et al*., 2001). In contrast, alendronate at concentrations higher than 10 µM markedly stimulated MMP-13 mRNA and immunoreactive protein in osteoblasts. The increase in the MMP-13 synthesis was mediated by an increase in the stability of collagenase transcripts (Varghese and Canalis, 2000).

3.5.6. Clinical use of bisphosphonates

BPs have become accepted as the most potent inhibitors of bone resorption clinically available and as a mainstay in the treatment of osteoporosis. Although many BPs have been investigated in human osteoporosis, most of the studies have been carried out with alendronete, etidronate and risedronate. Many well controlled studies have confirmed the efficacy of BPs in preventing the decrease in bone mineral density (BMD), as assessed by dual X-ray absorptiometry first in menopausal osteoporosis and then in other types of osteoporosis. BPs induce a marked decrease in bone turnover, when given in doses effective on BMD. Both bone formation and resorption are decreased (Fleisch, 2003). Alendronate (Black *et al*., 2000; Pols *et al*., 1999) and risedronate (Harris *et al*., 1999) have both been found to reduce vertebral and nonvertebral fractures. Intermittent cyclic therapy with etidronate has also been shown to reduce vertebral fracture risk (Cranney *et al*., 2001). The effect on fractures is probably both due to an increase in BMD and a decrease in bone turnover (Fleisch, 2003).

BPs are the treatment of choice for a variety of bone diseases in which excessive osteoclast activity is an important pathological feature, including Paget's disease and malignant bone disease. In humans, BPs inhibit tumor-induced bone resorption, correct hypercalcemia, reduce pain, prevent the development of new osteolytic lesions, prevent fractures and, consequently, improve the quality of life for the patients (Catterall and Cawston, 2003). Recently, in patients with operable breast cancer, clodronate has been reported to reduce the incidence of bone metastases (Diel *et al*., 1998; Powles *et al*., 2002).

3.5.7. BPs in inflammatory joint disease

3.5.7.1. General

As there is now compelling evidence that joint erosion in RA is crucially dependent on osteoclast activity, the rationale that BPs might be effective, not only for the management of systemic osteoporosis, but also as an useful adjunct in preventing local structural damage is well motivated. Indeed, in the rat adjuvant arthritis model both N-BPs and non-N-BPs were able to prevent bone erosions and to significantly reduce the inflammatory response (Frances *et al*., 1989; Österman *et al*., 1994). The fact that not only the bone resorption is decreased, but also the inflammatory reaction in the joint and paw itself diminished, suggests that mechanisms other than those acting on bone are at work, possibly involving the mononuclear phagocyte system (Fleisch *et al*., 2002).

With respect to collagen induced arthritis, clodronate modestly decreased clinical and histological signs of arthritis (Österman *et al*., 1995; Nakamura *et al*., 1996), whereas alendronate exacerbated the arthritis reaction (Nakamura *et al*.,1996). Sustained joint

swelling was reported after intra-articular administration of ibandronate in rat antigen-induced arthritis model (Richards *et al.*, 1999). Consistent with these *in vivo* pro-inflammatory effects, N-BPs have been shown *in vitro* to augment the release of TNF-α and IL –1β from LPS-stimulated monocyte/macrophage lineage cells (Richards *et al.*, 1999; Makkonen *et al.*, 1999). In a recent study, in which a collagen induced arthritis model was used, the potent third-generation bisphosphonate, zoledronic acid, reduced focal bone erosions, although a dose-dependent increase in paw swelling was observed. Thus, the most potent N-BPs, exerting greatest inhibitory effects on osteoclast mediated bone resorption, might prevent structural joint damage despite eventual exacerbation of clinical arthritis after N-BP administration (Sims *et al.*, 2004; Goldring and Gravallese, 2004).

3.5.7.2. Clinical trials in RA

The few controlled studies of BPs in RA, most of them with pamidronate, have shown variable results. In the study by Ralston *et al.* (1989) 40 patients were randomized to either 30 mg of pamidronate by monthly intravenous infusion or placebo for 48 weeks. No significant effects on disease activity or radiological progression was evident despite significantly reduced markers of bone resorption. In contrast, another study that examined the effects of a single intravenous infusion of placebo or 20 mg or 40 mg of pamidronate in 30 patients with active RA, showed significant clinical improvement and decline in erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) after the 40 mg dose (Eggelmaijer *et al*, 1994). This same group then examined 105 patients randomized to either 300 mg oral pamidronate daily or placebo for three years. No significant treatment group difference in disease activity or radiologic scores was evident (Eggelmeijer *et al.*, 1996).

3.5.7.3. Liposomal clodronate

If BPs are encapsulated in a lipososme, they are no longer sequestered by the skeleton; instead they are taken up by active phagocytic cells such as macrophages (Buiting *et al.*, 1996). In animal models of arthritis, encapsulated clodronate was found to decrease the numbers of macrophages and to reduce inflammation (Camillieri *et al.*, 1995; Kinne *et al.*, 1995; van Lent *et al.*, 1993). Promising results were recently obtained when clodronate liposomes were injected locally into human RA joint. The procedure was well tolerated and led to macrophage depletion and decreased expression of adhesion molecules in the synovial lining (Barrera *et al.*, 2000a).

4. THE AIMS OF THE STUDY

1. To evaluate the eventual anti-inflammatory effects of a single infusion of clodronate in patients with active RA.

2. To study the effects of clodronate infusion on salivary collagenase activity in RA patients.

3. To assess the effect of cyclic etridonate therapy on the progression of radiographic joint damage in RA patients in a two year study.

4. To examine the relationship of structural damage and markers of type I collagen metabolism and OPG in RA patients, and to evaluate the response of these bone biochemical markers on BP therapy.

5. To compare the effects of clodronate and pamidronate on MMP-9 production by activated human monocytes *in vitro.*

In more general terms, these aims could perhaps provide hints on the eventual value of BP treatment as an adjunct in anti-arthritic therapy.

5. PATIENTS AND METHODS

5.1. Patients and treatments

5.1.1. Patients and treatments in publications I and II

Twenty-six patients with RA according to the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria (Arnett *et al*., 1988) with the mean age of 51.8 years (range 36 to 61) were enrolled in a randomized, double-blind, placebo-controlled study at Department of Medicine, Helsinki University Central Hospital in year 1996. The study was approved by the Helsinki University Central Hospital ethics committee. All patients had active disease as defined by the presence of at least 3 of the following 4 criteria: (a) number of tender joints > 10 (maximum count 68); (b) number of swollen joints > 5 (maximum count 66); (c) duration of morning stiffness in joints ≥30 minutes; (d) ESR ≥28 mm/h or CRP ≥19 mg/l. Concomitant therapy with anti-rheumatic drugs and/or low-dose oral steroids (up to 10 mg/day prednisone or equivalent) was to be maintained unchanged one month prior to and during the study. Intra-articular or systemic glucocorticoid injections were not allowed within 2 weeks prior to or during the study. Patients with impaired renal function or serum calcium values outside the reference limits were excluded. Eligible patients were after their informed consent randomly allocated to receive a single iv. infusion of either clodronate 600 mg in 500 ml 0.9 % NaCl (n=14) or saline as placebo (n=12) over 5 h.

5.1.2. Patients and treatments in publications III and IV

Forty RA patients were enrolled in a randomized, parallel-group, controlled, open label and evaluator blinded (radiology, bone biochemistry) study between October 1998 and March 1999 at Helsinki University Central Hospital. Patients were eligible to the study if they met the 1987 ACR criteria for the classification of RA (Arnett et al.,1988), were 18 years of age or older, had a disease duration less than 5 years, had a minimum of 4 swollen joints (maximum count 66) at baseline and had elevated CRP (≥10 mg/l) or ESR (≥28 mm/h) either at baseline or within the previous 12 months. Patients previously treated with BPs and those who at baseline were judged to require BP treatment due to osteoporosis were excluded. Other exclusion criteria were pregnancy/breastfeeding, physical incapacity (Steinbrocker class IV) (Steinbrocker et al., 1949) and impaired renal function (serum creatinine > 115 μmol/l). The protocol and consent form were approved by the Helsinki University Central Hospital ethics committee. Informed consent was obtained from all patients.

After baseline assessment patients were randomly allocated to one of two treatment groups. Etidronate group (n=20) received intermittent cyclical oral etidronate disodium (a gift from Roche, Espoo, Finland) 400 mg daily for 2 weeks every 12–14 weeks (eight cycles total) in conjunction with antirheumatic therapy. Control group (n=20) received only antirheumatic therapy. Changes to the concomitant therapy with disease modifying antirheumatic drugs (DMARDs) and/or oral steroids could be made whenever considered appropriate throughout the study period in both groups.

5.2. Methods

5.2.1. Clinical evaluations (I, III, IV)

In the clodronate study disease activity was assessed at baseline and weekly during the following three weeks by number of swollen joints (maximum count 66), number of tender joints (maximum count 68), duration of morning stiffness (minutes), patient overall assessment of current disease activity on visual analogue scale (VAS) of 0–100 mm and doctor's estimation of patients condition on VAS.

In the etidronate study patients were assessed by the same clinician at baseline and 24 months. The following disease variables were measured: number of tender and swollen joints (28-joint count), patient overall assessment of current disease activity on VAS of 0–100 mm and the modified disease activity score (DAS28) using 28 joint counts (van Gestel *et al.*, 1998).

5.2.2. Radiographic evaluation (III, IV)

Radiographs of hands and feet were taken at the entry to the etidronate study and at 24 months and were scored in a chronological order according to van der Heijde's modification of Sharp's method (van der Heijde, 1999b) by one experienced reader who was unaware of the treatment assignments. The Sharp/ van der Heijde method includes 16 areas for erosion and 15 for joint space narrowing in each hand. The erosion score can range from 0 till 5. Joint space narrowing is combined with a score for (sub)luxation and scored with a range from 0 to 4. In feet 10 metatarsophalangeal joints and the two interphalangeal joints of the big toes are included. The maximum erosion score per joint in feet is 10, i.e. 5 at each side of the joint. The maximum erosion score of all joints in both hand is 160 and in both feet 120; the maximum score for joint space narrowing in all joints of both hands is 120 and in both feet 48. Summation of erosions and joint space narrowing gives the so called "total score" of hands and/or feet (van der Heijde, 1999b).

5.2.3. Blood chemistries and bone markers (I, II, III, IV)

In the etidronate study serum samples for bone biochemistry were collected at baseline and at 24 months and stored at –20 °C. In the clodronate study laboratory assessments were performed at baseline and weekly during the following three weeks.

5.2.4. Salivary samples (II)

As repeated joint aspirations would be impractical, we chose to collect serial salivary samples to assay collagenase activity *in vivo*. Samples were collected at baseline, after termination of the drug infusion (within 30 minutes), and then weekly during the following three weeks. Subjects first rinsed their mouth thoroughly with water and then chewed paraffin. Stimulated saliva was collected over five minutes. Immediately after the collection of the saliva the samples were centrifuged at 1000 g for five minutes and the supernatants were frozen at –20 °C until analyzed.

5.2.5. Clodronate concentration in saliva (II)

Disodium clodronate concentrations in saliva samples was analyzed by capillary chromatography with mass-selective detection (HP 5970). A stabile isotope -labeled analog of clodronate was used for the internal standard during the analytical process. The quantification limit for disodium clodronate in saliva was 10 ng/ml. Samples that had been collected in the clodronate group (n =14) immediately after drug infusion and at three weeks were analyzed.

5.2.6. Measurement of collagenolytic activity (II)

Collagenolytic activity was measured against soluble native triple helical collagen type I monomers. Native type I collagen was extracted from human skin and further purified by selective salt precipitations at acidic and neutral pH. Salivary samples were assayed for collagenase activity by the quantitative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) laser densitometric method originally described by Turto *et al*. (1977). The salivary samples were incubated with soluble native 1.5 µM type I collagen at 22 °C for 48 hours. Incubation was stopped by addition of a modified Laemmli's buffer followed by immediate heating at 100 ºC for 5 minutes. Subsequently, the degradation products were separated by SDS-PAGE in 10 % cross-linked gels. The gels were stained with Coomassie brilliant blue and destained in 10 % acetic acid. The destained gels were quantified by densitometric scanning using the LKB ultrascan laser densitometric model 2202. The value representing $3/4 \times (\alpha A)$-chains was multiplied by 4/3, and its proportion of the total collagen in the sample was used to measure collagenase activity. Collagenase activity was expressed as molar amounts of collagen degraded per hour (Suomalainen *et al*., 1992).

5.2.7. Western blotting (II)

Polyclonal rabbit anti-human MMP-8 was characterized and kindly donated by Dr Jürgen Michaelis, Department of Pathology, Christchurch Medical School, New Zealand (Michaelis *et al*., 1990). The polyclonal rabbit anti-human MMP-1 was characterized and kindly donated by Dr. Henning Birkedal-Hansen, Department of Oral Biology, School of Dentistry, University of Alabama at Birmingham, AL, USA. The immunization procedure for preparation of the polyclonal rabbit anti-human MMP-13 was carried out as described elsewhere (Freije et al.,1994). The saliva samples were analyzed using Western blotting according to Towbin *et al*. (1979) and Burnette (1981), with slight modifications. Briefly, the saliva samples were mixed with Laemmli's buffer and boiled for 5 minutes before application to 8–10 % polyacrylamide gels. Proteins separated in the gels by electrophoresis were electrophoretically transferred to nitrocellulose membrane (Bio-Rad Laboratories). The nonspecific binding sites on the membranes were blocked with 3 % gelatin in 10 mM Tris-HCl, pH 8.0, 0.05 % Triton X-100, 22 mM NaCl (TTBS). The membranes were incubated in the primary antibodies (1:500–1:750 dilution in TTBS) overnight and then with secondary antibodies for one hour. The secondary antibody was alkaline phosphatase conjugated goat anti-rabbit IgG. The proteins were visualized with nitro blue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate solution. All antibody incubations were performed at +20 °C and the membranes were washed 4×15 minutes in TTBS between each step.

5.2.8. Isolation and culture of human monocyte/macrophages (V)

Human mononuclear cells were isolated from buffy coat cells from healthy volunteers over Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) at 700 g for 20 minutes at room temperature. The mixed mononuclear cell band was removed by aspiration and the cells were washed with Ca^{2+}/Mg^{2+} free 0.1 M phosphate buffered 150 mM saline, pH 7.4 (PBS), and centrifuged at 400 g for 5 minutes for 3 times. The mononuclear cells were resuspended to 50 ml serum-free macrophage medium (SFM, GIBCO) with 1 % penicillin/ streptomycin and seeded at $4–5 \times 10^6$ cells per well in 24-well plates. Monocyte/ macrophages were allowed to adhere for 1 h at $+37\ °C$ in a 5 % CO_2 incubator. Nonadherent cells were washed away with PBS. Fresh medium was added and the monocyte/ macrophages were treated for 20–24 hours with clodronate (3,10,30,100,300 and 1000 µM) (Leiras Pharmaceutical Co., Tampere, Finland), pamidronate (1,3,10,30,100 and 300 µM) (Novartis Pharma AG, Basel, Switzerland) or vehicle. After incubation the cells were washed free of drugs and SFM supplemented with 10 mg/ml lipopolysaccharide (LPS) (E.Coli, serotype 0127:B8, Sigma) was added to the wells. The cells were incubated for an additional 24 hours and the cell supernatants were collected and stored at –80 °C until quantified by ELISA. For mRNA measurements larger wells and higher cell numbers (10-15×10^6 cells/well) were used. The drugs were not washed away and the cells were harvested after a 4 hour LPS-induction.

Cell viability was evaluated with trypan blue dye exclusion test (Phillips,1973) from samples subjected to 20 hour exposure to BPs followed by a subsequent LPS-induction for an additional 24 hours.

5.2.9. MMP-9 ELISA (V)

Microtiter plates (Flow Laboratories, Irvine, Scotland) were coated with 100 µl of 5 µg/ ml human MMP-9 specific mouse monoclonal antibody (TNO-S22.2) (Hanemaaijer et al., 1998) in PBS overnight at 4 °C. After three washes in PBS containing 0.05% (v/v) Tween 20 (PBS-T), 100 µl of purified MMP-9 (Hanemaaijer et al., 1998) or cell supernatant was added. After overnight incubation at 4 °C, the plates were washed and incubated for 1 hour at 37 °C with 100 µl of biotin-labeled anti-MMP-9 polyclonal antibody (TNO-B21) (Hanemaaijer et al., 1998) diluted in PBS-T/EDTA containing 0.1% (w/v) casein (PBS-T/ EDTA/C) (0.8 µg/ml). After washing, bound polyclonal antibody was assessed by incubation with 100 µl of avidin/HRP (Pierce, Rockford, Ill, USA) at 1:10.000 in PBS-T/EDTA/ C. Non-bound conjugate was washed away after 1 hour at 37 °C, and the chromogen 3,3'5,5'-tetramethyl benzidine together with H_2O_2 was added. The reaction was stopped after 20 minutes with 2M H_2SO_4, and the absorption was measured at 450 nm in a Titertek Multiskan spectrophotometer (Flow Laboratories).

5.2.10. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (V)

Total RNA was isolated by using TRIzol reagent (Gibco). mRNA was isolated from total RNA using magnetic $(dT)_{25}$-polystyrene beads (Dynal, Oslo, Norway). 100 ng mRNA was used to prepare primary cDNA using $(dT)_{12–18}$ primers and SuperScript enzyme, followed by RNase H treatment (GibcoBRL). Quantitative PCR was run on 10 ng of first strand cDNA using 0.5 mM primers in LightCycler™ SYBR Green I PCR mix by LightCy-

cler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany). The identity of the product was verified by a melting curve analysis. Serial dilutions of cloned human MMP-9 PCR fragment in plasmid DNA were used to determine the copy number of the amplicon per 1000 ß-actin mRNA copies. Each individual sample was amplified at least two times.

5.2.11. Electrophoretic mobility shift assay (EMSA) (V)

Human monocytes were plated to 6-well plates (10×10^6 cells/well) and treated with 30 µM clodronate or pamidronate for 20 hours, whereafter 10 µg/ml of LPS was added without removing the drugs. The cells were then incubated for an additional 4 hours. After the drug and LPS treatment the cells were washed with PBS and scraped to 1 ml PBS. The cell pellets were frozen and stored at –70 °C for analysis. Nuclear protein isolation and EMSA assay were performed as described earlier in detail (Helenius et al., 1996). Briefly, nuclear proteins were isolated according to the modified protocol of Dignam et al. (1983). Protein-DNA binding assays were performed with 5 µg of nuclear protein. Double-stranded consensus and mutated oligonucleotides for NF-κB binding site were obtained from Promega and labeled with T4 polynucleotide kinase (Promega). Unspecific binding was blocked by the use of 2 µg of poly(dI-dC):poly(dI-dC) (Roche Applied Science) in 20 µl assay volume. After binding bound and unbound probes were separated in a native 4 % polyacrylamide gel. Signals were visualized with Storm 860 PhosphorImager (Molecular Dynamics) and pixel volumes of specific bands were calculated with Image-QuaNT 4.2a software (Molecular Dynamics).

5.3. Statistical analysis

In study I changes in continuous variables over time within and between the groups were analyzed by analysis of variance (ANOVA) for repeated measurements (SAS® 608, SAS Institute). Baseline comparisons for continuous variables were done by one-way analysis of variance. If assumptions of parametric methods were not met, the Kruskal-Wallis and Mantel-Haenszel tests were used.

In studies II–V statistical analyses were done using SPSS – 9.0 software. In study II comparisons between treatment groups were done by Student's t-test. In studies III and IV changes in response variables from baseline to 24 months were compared between treatment groups using ANOVA for repeated measurements, or Mann-Whitney U-test, where appropriate. Categorical data were compared by chi-square test. The relation between variables was measured by estimating the Pearson product moment correlation coefficient or by using Spearman's rank correlation coefficient (III, IV). In study V Mann-Whitney U-test was used for statistical analyses. The data was presented as mean ± SD if not otherwise stated. The significance level was set at $P < 0.05$.

6. RESULTS AND DISCUSSION

6.1. Effect of a single infusion of clodronate on clinical disease activity and bone biochemical markers (I)

All 26 enrolled participants completed the study. At baseline there were no significant group differences in disease variables (Table 2) between the active and placebo groups. Single infusion of clodronate (600 mg) had no effect, during the three week observation period, on indices of disease activity, including number of swollen joints, number of tender joints, patient's self -estimation of her condition, CRP and ESR (Table 2). The results suggest that clodronate does not have short-term anti-inflammatory effects in RA. One possible explanation to this negative result could be the poor lipophilicity of the drug. As a consequence, the concentration reached in the synovium is not sufficiently high to affect lining macrophages (Barrera *et al*., 2000a). The negative result may also be related to the type of administration, i.e. only single infusion was given to the patients.

Table 2. Efficacy variables (mean ± SD) in the placebo-treated (n =12) and clodronate-treated (n = 14) rheumatoid arthritis patients

	Study Week			
Variable	Week 0	Week 1	Week 2	Week 3
Swollen joint count				
Placebo	7.8 ± 3.0	7.9 ± 4.1	7.7 ± 5.0[*]	8.5 ± 6.6
Clodronate	10.7 ± 5.0	10.6 ± 4.2	10.8 ± 4.4	10.1 ± 4.6
Tender joint count				
Placebo	14.0 ± 10.2	12.8 ± 8.8	12.0 ± 10.4[*]	12.8 ± 10.9
Clodronate	13.6 ± 8.9	13.6 ± 9.0	13.8 ± 9.5	13.1 ± 11.0
Patients self-estimation of condition (VAS)				
Placebo	40.7 ± 20.9	49.8 ± 24.8	38.9 ± 23.9[*]	48.9 ± 30.8
Clodronate	37.5 ± 17.6	41.6 ± 19.8	43.2 ± 19.4	43.7 ± 20.8
CRP (mg/l)				
Placebo	41.8 ± 28.5	46.3 ± 32.5	43.8 ± 35.2[*]	47.6 ± 39.2
Clodronate	41.6 ± 36.9	45.1 ± 41.5	50.9 ± 41.7	49.5 ± 39.7
ESR (mm/h)				
Placebo	36.9 ± 18.7	37.8 ± 19.5	36.5 ± 16.9[*]	40.6 ± 23.6
Clodronate	44.6 ± 25.5	48.4 ± 25.6	53.9 ± 29.5	45.8 ± 26.5

[*] Usable data were available for 11 patients

Clodronate treatment induced a statistically significant, jet only marginal decline in PICP and osteocalcin (Table 3). This modest effect, despite the high intravenous dose administered, probably relates to the choice of bone biochemical markers: due to the strong coupling of bone formation and degradation PICP and osteocalcin are expected to react to diminished bone resorption. However, if the indices of bone formation are used as surrogate markers, the drug effect cannot be detected in full only three weeks after infusion (Delmas, 1990). Assays to measure serum NTx and CTx were not available at the time of the study. Measurement of markers of collagen degradation including ICTP would probably have added to the information from this trial.

Table 3. Markers of bone metabolism in the placebo-treated (n =12) and clodronate-treated (n = 14) rheumatoid arthritis patients. Median (interquartile range)

	Study week			
Variable	Week 0	Week 1	Week 2	Week 3
S-osteolcalcin (µg/l)				
Placebo	3.8 (3.4–4.8)	3.7 (3.2–4.9)	4.0 (2.8–5.1)[*]	3.8 (2.9–4.6)
Clodronate	3.8 (3.0–4.7)	3.5 (2.7–4.5)	3.4 (2.9–4.6)	3.5 (2.6–4.2)[a, b]
S-PICP (µg/l)				
Placebo	112.5 (90.0–125.0)	98.0 (90.5–124.5)	115.5 (101.0–145.0)[*]	118.5 (100.0–164.5)
Clodronate	107.5 (81.0–138.0)	108.0 (87.0–125.0)	104.5 (92.0–123.0)	102.5 (85.5–127.5)[a]

[*] Usable data were available for 11 patients
[a] $p < 0.05$ compared to placebo
[b] $p < 0.05$ compared to baseline

6.2. *In vivo* inhibition of human neutrophil collagenase activity (MMP-8) by clodronate (II)

Quantitative SDS-PAGE electrophoresis scanning of the analyzed saliva of the patients showed a decline of collagenase activity in clodronate treated patients when compared to controls. The between group difference was statistically significant at three weeks. However, the collagenase activity was slightly lower in the clodronate group already at baseline (Table 4). Concomitant Western blots using polyclonal antibodies specific to MMP-8, MMP-1, and MMP-13 revealed that MMP-8 (75 kDa) was the major collagenase present

Table 4. Salivary collagenase activity in treatment and placebo groups during three weeks

	Mean (SEM) collagenase activity (pmol/h)				
	baseline	5 hours	day 07	day 14	day 21
Treatment group	271.0 (48.7)	223.7 (24.3)	265.7 (39.5)	208.1 (24.0)	195.0 (14.7)
Placebo group	309.7 (42.0)	301.5 (35.5)	292.0 (68.0)	258.0 (38.3)	267.2 (29.7)
P-value*	0.57	0.08	0.74	0.27	0.04

* Two-sample t-test

in saliva of RA patients (Figure 4, one representative blot of five samples). The mean (±SEM) clodronate concentration in saliva was 0.42 (± 0.11) µM after termination of the drug infusion and 0.13 (± 0.03) µM at three weeks.

As clodronate concentrations measured in saliva were low, not exceeding the nanomolar range, the direct enzyme inhibition by the drug is not a plausible explanation for the decreased collagenolytic activity (Teronen *et al.*, 1999). The life cycle of PMNs consists of maturation in the bone marrow (approximately 7–14 days) followed by the transit in blood for hours before migration into tissues, where their life span continues for 1–2 days (Bainton, 1975). Neutrophil collagenase MMP-8 is synthesized during the myelocyte stage of neutrophil development to be stored in the secondary or specific granules, and no *de novo* synthesis takes place during the later stages of maturation. Thus, downregulation of MMP-8 synthesis at myelocyte stage within bone marrow could offer an explanation for
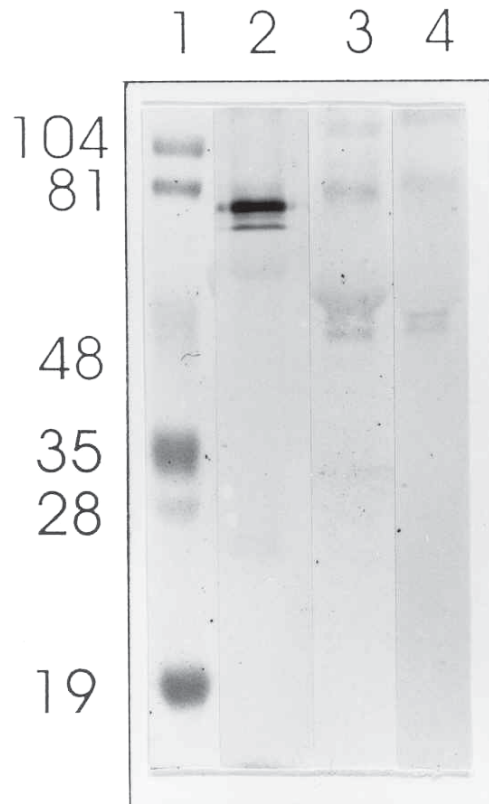
Figure 4. Western blot of saliva from rheumatoid arthritis patients. First lane contains molecular weight markers. Second lane demonstrates a lane blotted for MMP-8 (collagenase-2 or neutrophil collagenase) with an apparent molecular weight of 75 kD. Two minor bands with apparent molecular weights of 72 kD and 70 kD are also seen. The third lane demonstrates MMP-13 (collagenase-3; 60 kD for the major band) and the fourth lane MMP-1 (collagenase-1 or fibroblast collagenase; 57 kD for the major band). In general, MMP-8 gave strongest band of the three collagenases blotted, which suggests that this type of collagenase is the major collagenase in saliva from rheumatoid arthritis patients.

the decreased salivary collagenase activity measured three weeks after clodronate infusion. This novel mode of action of clodronate-mediated collagenase inhibition has to be considered hypothetical, however, and needs to be assessed further.

Both MMP-8 and -9 are found in high concentrations in the synovial fluid of RA patients (Tchetverikov *et al.*, 2004; Yoshihara *et al.*, 2000). The major cellular source of MMP-8 is PMN (Chatman *et al.*, 1990) whereas in rheumatoid joints MMP-9 has been immunolocalized to various cells including macrophages, chondrocytes and PMNs (Okada, 2001). However, the strong direct correlation between MMP-8 and MMP-9 in RA synovial fluid suggests that both MMPs are derived mainly from PMNs infiltrated into the synovial cavity (Yoshihara *et al.*, 2000). Furthermore, collagen degradation product hydroxyproline has been found to correlate with MMP-8 and MMP-9 in RA synovial fluid, implying that these proteases are likely to play part in the degradation of collagenous network in the joints (Tchetverikov *et al.*, 2004). Taken together, these findings suggest that inhibition of collagenase activity in saliva by clodronate may have relevance when considering its use as an adjunctive therapy in the treatment of RA.

6.3. Cyclical intermittent etidronate therapy in RA (III, IV)

6.3.1.General

All 40 enrolled participants completed the study except for one patient in the etidronate group who died of pneumonia during the second year of the study and was not included in the analyses except for the baseline correlation assessments. Baseline serum sample for bone biochemistry was not obtained from one patient in the etidronate group and she was excluded from the evaluations where these values were needed. Etidronate treatment was well tolerated. One patient stopped taking etidronate during the first cycle because of gastrointestinal complaints. She started taking etidronate again at the beginning of the second year and continued the medication to the end of the study. There were no statistically significant group differences among baseline patient characteristics (Table 5). DMARDs used by patients during the trial included hydroxychloroquine, sulfasalazine, methotrexate, gold, cyclosporin A, azathioprine, podophyllotoxin and leflunomide. At baseline 16 (84 %) patients in the etidronate group and 18 patients (90 %) in the control group received at least one DMARD. A combination of two or more DMARDs was received in the etidronate group by 8 (42 %) patients and in the control group by 10 (50 %) patients. At the end of the study the corresponding figures in the etidronate group were 18 (95 %) and 10 (55 %), and in the control group: 17 (85 %) and 11 (55 %). The mean prednisone doses at baseline and at 24 months were 3.3 mg/day and 2.4 mg/day, respectively, in the etidronate group and 2.8 mg/day and 3.0 mg/day, respectively, in the control group. The where no statistically significant differences between the groups in prednisone usage.

6.3.2. Effect of therapy on radiographic progression and clinical disease activity

In both treatment groups, there was a significant increase in all 3 mean radiologic scores. This progression was slightly less extensive in the etidronate treated patients, but the difference between the groups was not significant There were no differences among the treatment groups in the change between baseline and 24 months in variables of disease activity (Table 6).

Table 5. Patient characteristics at baseline (mean ± SEM if not otherwise stated).

	Etidronate group (n = 19)	Control group (n = 20)
Age, years	48.5 ± 2.4	52.6 ± 3.5
(range)	(22–66)	(26–78)
Males/females	2/17	2/18
Menopausal status (yes/no)	12/5	11/7
HRT/postmenopausal women	6/12	6/11
Disease duration, months	34.9 ± 3.5	36.2 ± 3.1
DAS28	4.0 ± 0.3	3.6 ± 0.3
CRP (mg/l)	26.1 ± 9.0	25.1 ± 4.5
ESR (mm/h)	29.2 ± 5.9	18.8 ± 3.9
Total radiographic score	18.0 ± 5.2	16.5 ± 3.3
Glucocorticoid therapy (%)	9 (47)	12 (60)

HRT = hormone replacement therapy; DAS28 = modified disease activity score; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

Table 6. Radiographic scores and variables of disease activity in the etidronate group (n=19) and the control group (n=20) during a 2 year study. Mean ± SEM.

Variable	Baseline	24 months	P
Total radiographic score			0.61
Etidronate	18.0 ± 5.2	23.7 ± 6.4	
Control	16.5 ± 3.3	23.4 ± 4.4	
Erosion score			0.64
Etidronate	14.8 ± 3.7	18.7 ± 4.3	
Control	12.9 ± 2.3	17.5 ± 3.1	
JSN-score[†]			0.79
Etidronate	3.2 ± 1.7	4.9 ± 2.3	
Control	3.7 ± 1.5	5.9 ± 1.8	
DAS 28			0.52
Etidronate	4.0 ± 0.3	3.3 ± 0.3	
Control	3.6 ± 0.3	3.2 ± 0.2	
CRP[†] (mg/l)			0.57
Etidronate	26.1 ± 9.0	7.5 ± 1.8	
Control	25.1 ± 4.5	9.0 ± 1.4	
ESR[†] (mm/h)			0.67
Etidronate	29.2 ± 5.9	21.6 ± 3.5	
Control	18.8 ± 3.9	17.1 ± 3.5	

† = Mann-Whitney test was applied for the analysis; JSN-score = joint space narrowing score; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate.

As joint counts and serum acute-phase response measurements were performed only at baseline and at 24 months, information concerning the clinical disease activity between these time points is limited. However, the significant decline in CRP suggests that disease activity diminished over the 2-year course of the study in both treatment groups (Table 6).

The dissociation between clinical synovitis, acute-phase responses and radiologic progression, suggested in the present as well as previous clinical studies (Paimela *et al.*, 1994; Mulherin *et al.*, 1996), is evident also in the more recent trials of TNF inhibitors which show that TNF inhibitors have a positive influence on radiographic progression even in those patients that do not have improvement of joint counts or CRP (Lipsky *et al.*, 2000; St Clair *et al.*, 2004; Klareskog *et al.*, 2004).

In the present study cyclical intermittent etidronate failed to retard the progression of radiographic damage in RA patients. It may well be that although the cyclic regimen is able to decrease the accelerated physiological bone remodeling associated with postmenopausal osteoporosis, continuous therapy is needed to halt the pathologic bone remodeling leading to local bone erosions in RA. Our group has now started a trial with continuous oral clodronate to assess this question.

6.3.3. Response of bone biochemical markers to therapy and relation to radiographic progression (III, IV)

6.3.3.1. Markers of type I collagen metabolism

The levels of PINP and ICTP declined in the etidronate group, but increased in the control group (Table 7). The differences between the groups in serum PINP and in serum ICTP level changes were significant. Also serum NTx slightly decreased in the etidronate group and slightly increased in the control group. The difference between the groups in serum NTx change was not significant.

Correlation coefficients between the changes observed in the bone collagen markers from baseline to 24 months and changes in radiologic scores are shown in Table 8. The change in serum NTx correlated significantly with the change in the total radiographic score and with the change in the erosion score, whereas the radiological progression was not related to changes in the other bone collagen markers. Changes in the levels of NTx, PINP and ICTP correlated significantly with each other. The changes in the bone collagen markers did not correlate with the changes in any of the disease activity measures (ESR, CRP and DAS28). In the control group, but not in the etidronate group, the change in serum NTx correlated with the change in the erosion score (r = 0.48, P = 0.034).

At baseline no significant correlations were found in the total study population (n = 39) between the markers of bone collagen metabolism and baseline disease characteristics or with the future radiographic progression of the joint damage. At study termination serum NTx, but not the other markers of bone collagen metabolism, correlated with the erosion score (r = 0.42, P = 0.008) and with the total radiologic score (r = 0.38, P = 0.018) in the total study population (n =39). In the control group (n=20) NTx correlated with erosion score (r = 0.63, P = 0.003) and with total radiologic score (r = 0.56, P = 0.01).

Table 7. Biochemical serum indices of bone metabolism at baseline and changes at 24 months in the etidronate group (n=18) and the control group (n=20).

Variable	Baseline	Change at 24 months	P
PINP (µg/L)			0.001
Etidronate	36.3 ± 4.5	–9.0 ± 3.2	
Control	35.1 ± 3.4	15.4 ± 6.1	
ICTP (µg/L)			0.04
Etidronate	3.3 ± 0.4	–0.5 ± 0.2	
Control	3.3 ± 0.4	0.6 ± 0.6	
NTx (nmol BCE/l)			0.18
Etidronate	15.2 ± 1.0	–0.7 ± 0.6	
Control	15.9 ± 1.4	1.5 ± 1.4	
OPG (pg/ml)			0.91
Etidronate	74.3 ± 5.3	3.7 ± 3.7	
Control	75.9 ± 4.6	2.3 ± 3.1	

PINP = N-propeptide of type I procollagen (reference range men: 20–76 µg/L; women: 19–84 µg/L); ICTP = crosslinked C-telopeptide of type I collagen (reference range: 1.6–4.6 µg/ml); OPG = osteoprotegerin. NTx = N-telopeptides of type I collagen (reference range men: 8.1 – 24.8 nmol BCE/l; women: 7.7–19.3 nmol BCE/l); BCE = bone collagen equivalent.

NTx reflects directly osteoclastic cathepsin K-dependent matrix degradation but it can not be generated by MMPs (Atley *et al*., 2000; Sassi *et al*., 2000). Thus, the significant correlation between change in serum NTx and increase in erosion score provides biochemical evidence for the concept that osteoclast is the principal cell type and cathepsin K the main protease responsible for the focal bone resorption in inflammatory arthritis. Similar conclusions were made from a recent cross-sectional study of RA, in which serum cathepsin K levels significantly correlated with radiological destruction but not with serum CRP levels (Skoumal *et al*., 2005). In the present study the lack of significant correlation between NTx and cartilage damage (joint space narrowing) was perhaps anticipated since in cartilage collagen type II predominates and cartilage collagen degradation is thought to be mediated mainly by MMPs (Okada, 2001; Tchetverikov *et al*., 2004).

The significant decrease in serum PINP in the etidronate treated patients is in line with previous observations which indicate that changes in PINP significantly predict efficacy of antiresorptive therapy in postmenopausal women (Saarto *et al*., 1998; Reginster *et al*., 2004). The reason for the marked increase in serum PINP in the control group is unclear, but may be related both to the small sample size and to the fact that a great proportion of patients in this study were postmenopausal women and, thus, in risk of loosing bone rapidly.

Although PINP was not related with the local bone resorption in joints, the strong correlation between a marker of bone formation (PINP) and markers of bone collagen degradation (ICTP, NTx) indicates that coupling of general bone resorption and bone formation is conserved in patients with rheumatoid arthritis.

6.3.3.2. OPG

At baseline and at study termination (Figure 5) serum OPG correlated significantly with age (r = 0.45, P = 0.003 and r = 0.56, P = 0.0002, respectively), but not with markers of type I collagen metabolism, indices of disease activity or radiological scores. Etidronate had no effect on circulating OPG levels (Table 7). The change in serum OPG was not related to changes in markers of type I collagen metabolism or progression of radiologic joint damage during the two year study (Table 8). At baseline serum OPG (mean ± SEM) was higher in patients receiving 5–10 mg/day prednisone (82.8 ± 4.0 pg/ml, n = 16) compared to those receiving < 5mg/day or with no prednisone (69.7 ± 4.7 pg/ml, n = 23) (P = 0.05). At baseline serum OPG correlated positively with prednisone dose (Spearman's r = 0.36, P = 0.02).

The results suggest that serum OPG measurement, perhaps because of the complexity of the regulation of the OPG, may be difficult to utilize in the evaluation of antiresorptive therapy. However, the study had some potential limitations. The number of patients was small and the study did not include serial BMD measurements. Determination of RANKL serum levels might have added to the information from this trial. The RANKL-to-OPG ratio might show greater utility for the assessment of the efficacy of antiresorptive therapy than serum OPG alone.
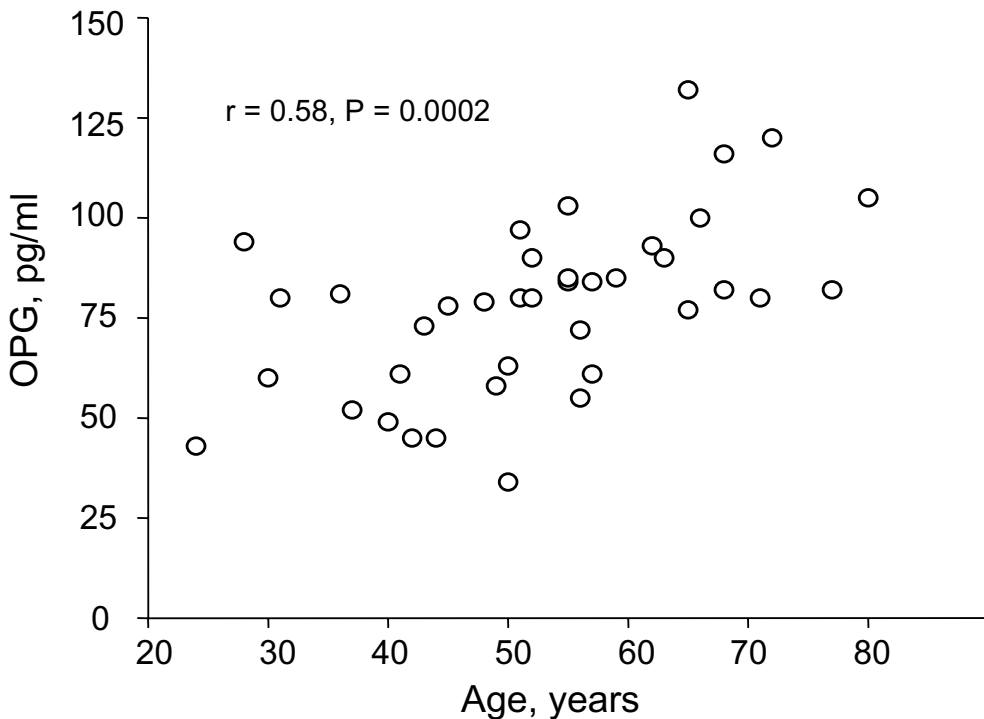

Figure 5. Correlation between serum levels of OPG and age in 39 RA patients.

Table 8. Correlation matrix showing relationships among changes in levels of markers of bone metabolism and radiologic scores during a 2 year study in total study population (n = 38). Pearson product moment correlation coefficient (r) was calculated for normally distributed parameters and Spearmans's rank order correlation (r_s) for non-normally distributed data (ICTP, JSN).

	ICTP	PINP	NTx	OPG
Total SHS	$r_s = 0.25$ P = 0.13	r = 0.22 P = 0.19	r = 0.35 P = 0.03	r = 0.02 P = 0.90
Erosion score	$r_s = 0.17$ P = 0.31	r = 0.25 P = 0.13	r = 0.41 P = 0.01	r = 0.03 P = 0.85
JSN-score	$r_s = 0.27$ P = 0.11	$r_s = 0.23$ P = 0.17	$r_s = 0.30$ P = 0.066	$r_s = 0.01$ P = 0.93
ICTP	—	$r_s = 0.48$ P = 0.002	$r_s = 0.56$ P = 0.0002	$r_s = 0.004$ P = 0.98
PINP		—	r = 0.67 P < 0.0001	r = - 0.08 P = 0.64
NTx			—	r = - 0.06 P = 0.72

See Table 7 for abbreviations.

Age was the only parameter that was significantly related to serum OPG in our cohort of RA patients (Figure 5). Age-related increase of OPG, found in most previous studies, possibly represents a compensatory mechanism against age-dependent bone loss. Decreased clearance of OPG with age, however, has been proposed as an alternative explanation to this finding (Khosla *et al*., 2002).

Glucocorticoids inhibit OPG production in human osteoblastic lineage cells (Hofbauer *et al*.,1999). Sasaki *et al*. (2002) reported that high-dose systemic glucocorticoid therapy with the mean initial dose of 43.8 mg/day led to decreased OPG serum levels. OPG levels remained suppressed after six months of therapy when the mean daily prednisone dose had been tapered to 16.5 mg. These findings are in contrast to our results with lower dose prednisone and the observation that serum OPG is elevated in patients with Cushing's syndrome (Ueland *et al*., 2001). Possibly higher prednisone doses than 10 mg/day are needed to inhibit OPG production *in vivo*. At lower doses other mechanisms lead to a negative balance between bone formation and resorption (Dempster, 1989) and the elevated OPG levels may represent an insufficient counter-regulatory mechanism to prevent bone loss.

6.4. Regulation of MMP-9 in activated human monocyte/ macrophages by BPs (V)

MMP-9 concentrations in unstimulated (negative control) and LPS-stimulated monocyte culture supernatants (n=4) were at 24 hours 13.6 ± 10.2 ng/ml and 51.3 ± 43.7 ng/ml, respectively. Pretreatment for 20–24 hours with clodronate induced an inhibition of the LPS-stimulated MMP-9 protein levels in a concentration-dependent manner (Figure 6). This effect was significant at 30–1000 μM concentrations. High concentrations (100 and 300 μM) of pamidronate also inhibited cellular secretion of MMP-9. In contrast, low concentrations of pamidronate enhanced MMP-9 secretion (Figure 6). The effect of the drugs on MMP-9 secretion from cells not stimulted with LPS was studied in one experiment that was not repeated. Pamidronate at 3–100 μM, but not clodronate, induced a many-fold increase on MMP-9 secretion. This effect was strongest at a concentration of 30 μM of pamidronate.

MMP-9 mRNA levels were relatively stable in the presence of clodronate. In contrast, pamidronate at 30 mM to 300 mM caused a 5- to10-fold increase in MMP-9 mRNA
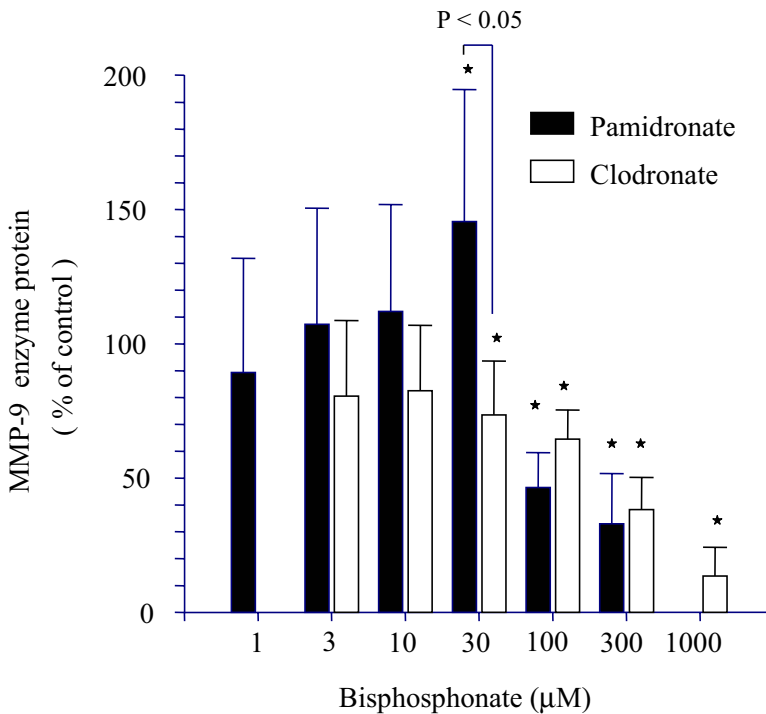

Figure 6. The effect of pamidronate (black columns) and clodronate (white columns) on MMP-9 secretion from LPS-stimulated adherent human monocytes. $1–1.5 \times 10^6$ cells were preincubated with drugs or vehicle. After an overnight incubation, the cells were washed free of drugs and, thereafter, incubated for additional 24 h with LPS (10 μg/ml) in serum-free macrophage medium. The LPS-stimulated cells not pretreated with bisphosphonates (control) produced 51.3 ± 43.7 ng/ml MMP-9. Bars represent the mean $\pm$ SD from four independent experiments. *, $P < 0.05$ versus control.

(Figure 7). The mRNA increase was significant in pamidronate treated cells (n = 12) compared to clodronate (n = 12) (422 ± 443 % vs. 94 ± 46 %, P < 0.001). LPS did not increase MMP-9 mRNA at 4 hours although it caused a significant increase in DNA binding of NF-kB in the EMSA-assay (Figure 8). On the other hand pamidronate, at concentrations that caused a many-fold increase in MMP-9 mRNA, did not increase the amount of NF-kB available for DNA binding (Figure 8).

Figure 7. The effect of pamidronate (triangles) and clodronate (squares) on MMP-9 mRNA expression in adherent human monocytes. $2–3 \times 10^6$ cells were preincubated with drugs or vehicle for 20–24 h and, thereafter, for 4 h with LPS (10 µg/ml) in serum-free macrophage medium. Data are mean ± SEM from two independent experiments performed in duplicates. The average MMP-9 mRNA expression in LPS-stimulated cells not pretreated with bisphosphonates (control) was 151 copies per 1000 ß-actin mRNA copies.
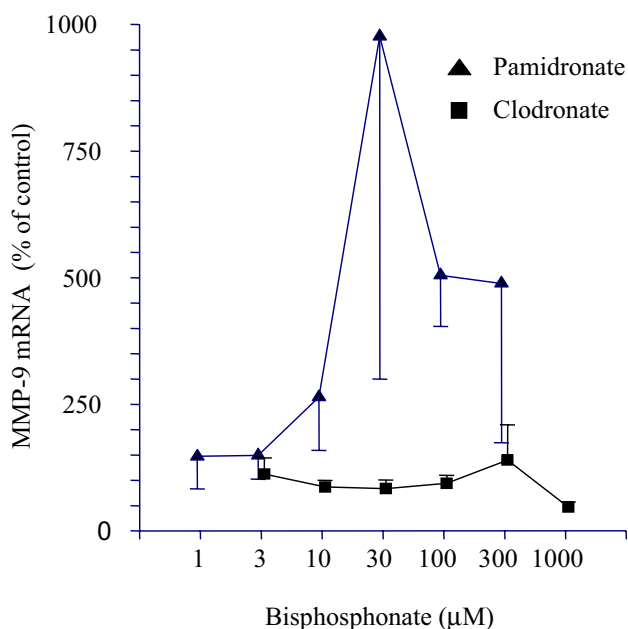

Figure 8. The effect of clodronate and pamidronate on the DNA binding activity of NF-κB in LPS-stimulated adherent human monocytes. 10×10^6 cells were preincubated with drugs or vehicle for 20–24 h and, thereafter, for 4 h with LPS (10 mg/ml) in serum-free macrophage medium. Lanes 1 and 2, untreated control cells; lanes 3 and 4, LPS-induced cells (not pretreated with BPs); lanes 5 and 6, clodronate 30 µM; lanes 7 and 8, pamidronate 30 µM. The same results were obtained from two parallel studies. The intensity of the bands was determined with Storm 860 PhosphorImager and values show the pixel volumes ($\times 10^3$) of specific bands calculated with ImageQuaNT 4.2a software.
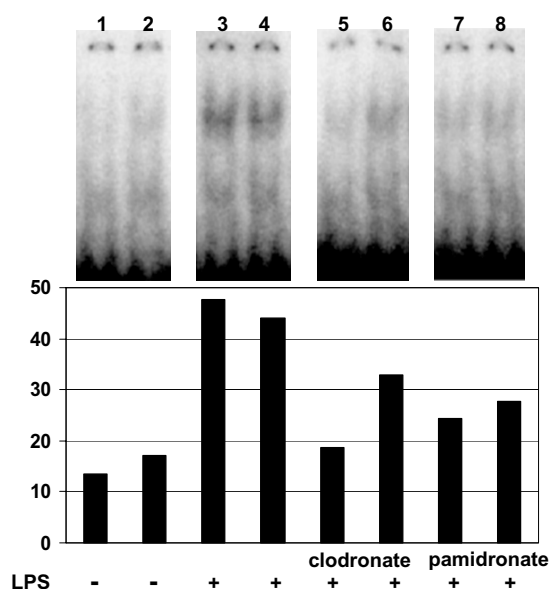
Trypan blue dye exclusion test disclosed that the viability of the monocytes was not reduced by the drugs except that the highest concentration of clodronate (1000 mM) was somewhat toxic with the cell viability being approximately 70% of that of the controls.

BPs may directly inhibit the proteolytic acitivty of MMPs through chelation of divalent cations (Teronen et al., 1999). In the present work the drugs were washed away prior to the MMP induction. Furthermore, the mono- and polyclonal antibodies used in ELISA recognize both active and latent MMP-9 as well as MMP-9 complexed to TIMPs (Hanemaaijer et al., 1999). Thus, the observed decline in MMP-9 concentration in culture supernatants was probably due to diminished monocyte-mediated MMP-9 production.

Recently alendronate at 10–100 µM was shown to up-regulate MMP-13 in osteoblasts by prolonging the half-lifes of collagenase transcripts (Varghese and Canalis, 2000). Accordingly, increased mRNA stability could explain the elevated MMP-9 mRNA levels in pamidronate treated monocyte/macrophages. Since MMP-9 is a NF-κB regulated gene (Farina, 1999), the finding that pamidronate did not increase the NF-κB binding of DNA is in accordance with the suggestion that posttranscriptional mechanisms lead to an increase in MMP-9 mRNA in pamidronate treated cells. Despite elevated message levels pamidronate, however, did reduce MMP-9 secretion at 100 to 300 µM. It is suggested that upon exposure to N-BP, two simultaneous and antagonizing events take place in monocyte/macrophages. The drug elevates MMP message levels, probably by increasing MMP mRNA stability. Secondly, it inhibits protein prenylation (Russel and Rogers,1999) and thereby a variety of cellular functions leading to diminished enzyme protein secretion at higher drug concentrations.

In the present study LPS had not increased MMP-9 mRNA at 4 hours despite a significant increase in the DNA binding of NF-κB. It could be that MMP-9 mRNA increase does not take place so early after LPS-stimulation and that it also seems to require endogenous TNF-α (Watari et al., 2000). Similarly, chemokine-induced MMP-9 production in peripheral blood monocytes occurs at 6–8 hours after stimulation, which is a late response compared with the stimulation of migration. This late response may be because leukocytes must first migrate through the endothelial cell layer. Only after migration into the sub-endothelial layer do they need MMP to degrade BM proteins (Robinson et al., 2002).

7. GENERAL DISCUSSION

7.1. Anti-inflammatory effects of BPs

Evidence accumulated in recent years strongly suggests that synovial macrophages play a major role in the initiation and maintenance of arthritis (Burmester, 1997). Macrophages are the major producers of TNF-α and other pro-inflammatory mediators in RA and the success of recent clinical trials with anti-TNF treatment (Lipsky *et al.*, 2000; Klareskog *et al.*, 2004) further suggests that synovial macrophages are important targets for therapy of RA. Previously pamidronate and clodronate have been demonstrated to have inhibitory effects *in vitro* on the secretion of proinflammatory cytokines from activated mouse macrophages (Pennanen *et al.*, 1995). Free pamidronate was approximately ten times more potent than clodronate in inhibiting TNF-α release but when encapsulated in negatively charged unilamellar liposomes the drugs were almost equipotent. Pamidronate is less water soluble than clodronate which property may contribute to its better diffusion into cells. Low lipid solubility could thus explain why intravenous clodronate, unlike pamidronate in the study by Eggelmeijer *et al.* (1994), did not suppress the disease activity in patients with RA.

Encapsulation to liposomes (van Lent *et al.*, 1998) or incorporation into albumin microspheres (D'Souza *et al.*, 1999) have been proposed as effective means to enhance the potency of clodronate as a macrophage suppressor in chronic inflammatory diseases. Recently the first human study on clodronate liposomes was published (Barrera *et al.*, 2000a). Ten RA patients scheduled for knee joint replacement received a single intra-articular dose of clodronate (mean dose 160 mg) encapsulated in liposomes. The procedure was well tolerated and resulted in a selective depletion of lining macrophages and a decline in the expression of adhesion molecules in the synovial lining layer. The down-regulation of adhesion molecules probably reflected a decrease in the local production of pro-inflammatory cytokines as judged by similar results obtained with TNF blocking treatment in patients with RA (Tak *et al.*, 1996). Intra-articular injection of free clodronate did not yield any changes in histological scores, which further underlines the fact that when administered as a free drug clodronate has little effect on other than bone tissue (Barrera *et al.*, 2000b).

7.2. Anticollagenolytic effects of BPs

Our finding that clodronate, and pamidronate at higher concentrations, inhibit cellular secretion of MMP-9 by human monocytes is in contrast to the report by Nakaya *et al.* (2000) that tilundronate does not affect MMP-1 and MMP-3 production by human periodontal ligament cells. BPs have been shown to have antiapoptotic effects on cells of the mesenchymal lineage, while promoting apoptosis of macrophages and osteoclasts (Plotkin *et al.,* 1999; Frith *et al.,* 2001). The different effects on MMP secretion by BPs in the two experiments could thus be related to the contrasting effects of BPs on these two different cell types. Naturally, MMP-9 compared to MMP-1 and MMP-3 may be differently regulated at the transcriptional and posttranscriptional levels.

The biphasic effect of pamidronate on MMP-9 secretion, demonstrated in this study, could explain some previous observations. Accordingly, low concentrations of N-BPs were shown paradoxically to enhance osteoclastic resoption *in vitro* (Sato *et al*., 1990; van der Pluijm *et al*., 1991). In animal models of metastasis, administration of N-BP was occasionally followed by enhanced soft organ metastases despite simultaneous inhibition of bone metastases (lower drug concentrations are reached in soft tissues compared to bone) (Sasaki *et al*., 1995; Stearns and Wang, 1996; Cruz *et al*., 1998). This effect could be related to N-BP induced MMP-9 expression, since MMP-9, produced by non-neoplastic inflammatory cells in the tumor vicinity, plays an important role in tumor invasion and metastasis (Nelson *et al*., 2000).

Clinical studies with broad-spectrum MMP inhibitors have demonstrated limited effect on joint destruction, and a range of side effects, indicating the need for development of selective inhibitors (Catterall and Cawston, 2003). Experimental evidence suggests an opposite role for MMP-2 and MMP-9 in the development of inflammatory arthritis. As the two enzymes have very similar specificities for matrix proteins, these opposite roles may be caused by differences in their non-matrix substrates. Indeed, several tissue-derived MMPs, MMP-2 in particular, cleave and inactivate MCP-3 (McQuibban *et al*., 2000) thus acting as chemokine antagonists, dissipating proinflammatory activities. In addition, at the sites of acute and chronic inflammation MMP-3 degrades active form of IL-1, leading to its inactivation (Schönbeck *et al*., 1998). In contrast, MMPs expressed by bone marrow derived leukocytes enhance the progression of RA (Itoh *et al*., 2002; van den Steen *et al*., 2002). MMP-9, in particular, can be seen as a tuner and amplifier of immune functions (Opdenakker *et a*l., 2001), and as a target for inhibition in inflammatory arthritis.

MMP inhibition by BPs *per se* has not shown specificity for individual enzymes. However, clodronate might, as suggested in this study, preferentially down regulate leukocyte-derived MMPs *in vivo*. This specificity, which is anticipated because of the high uptake of the drug in marrow bone, could be advantageous in the treatment of inflammatory arthritis.

7.3. Response of bone biochemical markers to therapy and relation structural damage

7.3.1. Markers of type I collagen degradation

In contrast to our results, previous studies that have examined ICTP levels in RA, have demonstrated a correlation between ICTP levels and the radiologic damage scores (Hakala *et al*., 1993; Paimela *et al*.,1994, Kotaniemi *et al*., 1994). These studies also showed a strong correlation between ICTP levels and CRP, ESR and joint swelling score, and the patients had higher overall disease activity compared to our study. Thus, the suppressed state of disease activity could explain the lack of correlation between ICTP and radiographic scores in our RA patient cohort. However, the radiographic damage worsened significantly during the follow-up, and this change was positively related to increase in serum NTx.

The observation that radiologic progression may occur during persistent remission has led to the hypothesis that synovitis and joint destruction are two different and independent processes and that a general cartilage thinning is a response to persistent synovitis, where-

as erosions are primarily caused by pannus (which may contribute little to joint swelling) (Molenaar *et al*., 2004; Kirwan, 2004). All the above mentioned studies examining ICTP in RA used a scoring system (Larsen method) that combines erosions and joint space loss into a single overall score and that was in this respect dissimilar to the system we used. Thus, the difference in the radiographic scoring systems could contribute to the divergent outcomes.

Taken together, one can hypothesize that in RA ICTP is primarily a marker of collagen breakdown that is associated with active synovitis, may be extracellular in nature and involves collagen type I degradation by MMPs (Risteli and Risteli, 1999). Changes in NTx, on the other hand, reflect local bone loss that may proceed during minimal or absent synovitis, and resembles cathepsin K-mediated osteoclastic bone resorption. Our results suggest that serum NTx could serve as a marker for progression of erosions even during periods of low disease activity.

7.3.2. OPG

In a study from Japan (Yano *et al*., 1999) conducted in 186 postmenopausal women, OPG serum levels correlated positively with biochemical markers of bone turnover and negatively with bone mineral density (BMD). Although these findings were not confirmed by Szulc *et al*. (2001) in a cohort of 252 healthy men, a generally accepted hypothesis is that OPG levels may increase with increases in bone turnover, possibly as a compensatory mechanism (Khosla *et al*., 2002). Consequently, etidronate with its demonstrated efficacy in the treatment of postmenopausal (Storm *et al*., 1990) as well as in steroid-induced (Adachi *et al.,*1997) osteoporosis could have been expected to decrease serum OPG. In contrast to this expectation, serum OPG slightly increased in the etidronate treated patients, despite the probable inhibition of bone resorption in this group as indirectly suggested by a significant decline in serum PINP (Saarto *et al*., 1998). However, i*n vitro* BPs increase OPG expression in human osteoblasts (Viereck *et al*., 2002), and therefore, a direct effect of etidronate on OPG production in bone tissue can not be excluded.

Recently Ziolkowska *et al*. (2002) reported normalization of elevated serum OPG levels in RA patients after treatment with anti- TNF-α therapy, but, similar to our results, they did not find any significant correlation between clinical disease activity (DAS28) and serum OPG. Here we also describe a lack of correlation between serum OPG and radiographic disease progression.

7.4. Inhibition of structural damage in RA by BPs

Thus far the largest study to evaluate the effect of BP treatment on structural damage in RA examined 105 patients randomized to either 300 mg oral pamidronate or placebo for three years (Eggelmeijer *et al*., 1996). Pamidronate significantly increased BMD of the femoral neck, forearm and lumbar spine, but did not retard the progression of radiographic joint damage. There is now strong evidence available to support the concept that normal osteclastic bone resoprtion is mainly cathepsin K dependent, whereas MMPs may become important in clinical states involving enhanced pathological bone breakdown such as arthritis (Garnero *et al*., 2003; Hakala *et al*., 1993). Thus, the finding that N-BPs increase MMP expression (Varghese *et al*., 2000; present study) may, together with the inflamma-

tory responses associated with N-BP treatment (Goldring and Gravallese, 2004), explain why pamidronate did not prevent the progression of local bone erosions although it had beneficial effect on systemic osteoporosis (Eggelmeijer *et al*., 1996).

It remains to be seen, whether the most potent N-BPs that exert strongest inhibitory effects on osteoclast mediated bone resorption, might prevent structural joint damage, even in instances they exacerbate clinical arthritis (Sims *et al*., 2004; Goldring and Gravallese, 2004).

8. CONCLUDING REMARKS

Significant association between an increase in serum NTx and worsening of erosion score provides biochemical evidence for the concept that osteoclast is the principal cell type responsible for the local bone resorption in RA, and suggests that monitoring changes in serum NTx may be useful to predict efficacy of treatment on progression. Serum OPG, on the other hand, probably due to the complexity of its regulation, did not emerge as a feasible surrogate marker for structural damage in RA.

The lack of suppressive effects of BP treatment on synovial inflammation in the present study may be related to the low circulating levels achieved with doses and regimes typically used for the treatment of osteoporosis. In RA, the presence of subchondral bone marrow inflammation and high rate of local bone turnover facilitates concentration of the BP drugs in subchondral bone to levels that could be anticipated to inhibit formation of focal bone erosions. The negative outcome in this respect may be due to the fact that the least potent and cyclically administered BP, i.e. etidronate, was used. Clodronate is more potent and, unlike etidronate, can be administered continuously. Downregulation of leukocyte-derived MMPs, as suggested in this study, could represent an additional anti-arthritic mechanism of clodronate.

Our group has now started a long term trial to evaluate the ability of clodronate to prevent structural damage in RA. As significant correlations are found between synovial fluid and serum MMP-9 and MMP-8 levels in RA (Tchetverikov *et al*., 2004), analysis of systemic levels of these MMPs in the ongoing study might add evidence for a chondroprotective effect of clodronate in RA. An enzyme immunoassay for cathepsin K is now available (Skoumal *et al*., 2005), and it would be of interest to evaluate the effects of antiresorptive therapy on this parameter as well.

The present study had some potential limitations. In the long-term trial with etidronate the number of patients was small considering that radiological progression was the primary outcome measure. As BMD measurements were not performed, conclusions about the effects on general bone loss were indirect, based on biochemical serum markers. In the *in vitro* study the use of human monocytes from healthy volunteers (instead of a cell-line) was associated with substantial variability between experiments affecting the evaluation of the effects of drugs on MMP-9 production.

The finding that the N-BP pamidronate upregulates MMP-9 expression in activated human monocytes may, together with the inflammatory responses associated with N-BP treatment, explain why pamidronate in a previous RA trial did not suppress formation of focal bone erosions. Recent preclinical studies suggest, however, that the most potent N-BPs, exerting greatest inhibitory effects on osteoclast mediated bone resorption, might prevent structural joint damage, even in instances they exacerbate clinical arthritis (Sims *et al*., 2004; Goldring and Gravallese, 2004). Alternative therapeutic approaches for inhibiting osteoclast mediated bone resorption that could potentially have therapeutic applications to rheumatoid arthritis include cathepsin K inhibitors (Wang *et al*., 2004), Fc-OPG fusion protein (Bekker *et al*., 2001; Kong *et al*., 1999) and a specific fully human monoclonal antibody to RANKL, which prevents RANKL binding to RANK (Bekker *et al*., 2004).

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