

# Cartilage oligomeric matrix protein (COMP) is modified by intra-articular liposomal clodronate in an experimental model of arthritis

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## Abstract

### Objective

High-dose liposomal bisphosphonates exert apoptotic effects. This work studies the chondroprotective and anti-inflammatory properties of intra-articularly administered low-dose, non-cytotoxic liposomal clodronate.

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### Methods

Antigen induced arthritis in rabbits was treated with intra-articular injections of liposomal clodronate. Drug effects on cartilage oligomeric matrix protein COMP was assessed using immunohistochemistry and morphometry of synovial membrane and hyaline articular cartilage.

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### Results

COMP remained close to normal in liposomal clodronate treated superficial articular cartilage compared to a significant loss of COMP in arthritis controls treated with empty liposomes. The middle and deep layers of the hyaline articular cartilage were characterized by highly increased COMP expression in liposomal clodronate treated AIA joints compared to controls. In contrast to cartilage, synovial COMP expression was slightly decreased as a result of liposomal clodronate treatment.

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### Conclusion

Low-dose, non-cytotoxic liposomal clodronate exerts a dichotomous effect on synovial membrane and articular cartilage COMP in the AIA model. COMP is a useful inflammation marker in the synovial tissue, but it also contributes to the structural integrity of the hyaline articular cartilage forming bridges between type II and IX collagens. Enhancement of COMP in clodronate treated AIA cartilage suggests a chondroprotective and anti-inflammatory effect in the inflammatorily damaged and mechanically strained cartilage.

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### Key words

Cartilage, arthritis, AIA, COMP, clodronate.

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## Introduction

Diseases affecting articular cartilage are usually treated with analgesics or non-steroidal anti-inflammatory drugs. Intra-articular glucocorticosteroids are used for acute exacerbations of osteoarthritis (OA) and rheumatoid arthritis to inhibit local inflammation, but it has also been known for a long time that they can also lead to pronounced articular cartilage proteoglycan loss (1, 2), in particular if injected into healthy joints (3, 4). Therefore, alternative modes of management with chondroprotective systemic and intra-articular therapies have been a focus of interest. It has been found that bisphosphonates prevent damage of the cartilage upon systemic administration in rabbits (5) and humans (6).

Chondroprotective effects can be analyzed using experimental models and intra-articular administration. In antigen-induced arthritis (AIA) in rabbits, a low-dose, non-cytotoxic, intra-articular liposomal clodronate clearly reduced proteoglycan loss from the superficial zone of the cartilage (7). This was demonstrated using Safranin O staining and microdensitometric evaluation. Loss of superficial zone proteoglycans is a sensitive, but also reversible indicator of slight cartilage damage, whereas cartilage oligomeric protein (COMP) is released from cartilage in response to IL-1 $\alpha$  at an intermediate time between proteoglycans and type II collagen degradation (8).

Whereas proteoglycans are water-binding matrix components, important for the elastic modulus of the cartilage matrix and elastohydrodynamic surface properties of the cartilage, COMP is an integral structural component of the cartilage matrix (9-12), which has also been used as a serum marker for disease activity and progression of OA (13, 14). COMP plays an important role in extracellular matrix assembly and matrix-matrix protein interactions (15). COMP binds to type II and type IX collagen bridging collagen fibrils (10, 12). It is important for the maintenance of the structural integrity and mechanical properties of cartilage under stress. It also plays a role for the

storage and delivery of hydrophobic hormones like retinol, retinoic acid and vitamin D (16) with implications for cartilage repair. Thus, in contrast to a reversible loss of cartilage proteoglycans and metachromatic staining, it may be more difficult for the cartilage to recover from loss of COMP. The aim of this study was to investigate the effect of low-dose, non-cytotoxic, intra-articularly delivered (targeted) liposomal clodronate on the structural integrity of the cartilage using COMP expression as its marker. Liposomal clodronate treated AIA was compared to AIA treated with empty liposomes and to healthy control joints.

## Materials and methods

### *Preparation of clodronate and calcein liposomes*

110 mM clodronate (Leiras Pharmaceutical Co., Tampere, Finland) was packaged into liposomes. Liposomes were prepared by reverse-phase evaporation from distearoylphosphatidylglycerol, distearoylphosphatidylcholine and cholesterol (Orion Farmos Co., Turku, Finland) (17). Clodronate was measured by phosphorus assay (17). Liposomes were prepared under aseptic conditions. The liposomes are referred to as liposomal clodronate (liposomes containing clodronate) and empty liposomes, which correspond to the treatment and placebo groups, respectively.

### *Induction of AIA*

Animal experiments were carried out with the approval of the Local and the County Ethical Committees. Hypnorm (Janssen Pharmaceutica, Beerse, Belgium) 0.05-0.1 ml/kg was used for animal sedation. New Zealand white rabbits were immunized on three occasions with two weak intervals. Emulsified mixture of 5 mg of ovalbumin (Sigma, St. Louis, MO) and Complete Freund's Adjuvant (Sigma) containing 1 mg of *Mycobacterium tuberculosis* was injected subcutaneously into multiple sites of the shaved interscapular region. Tertiary immunization was done by intramuscular injection. Development of delayed-type hypersensitivity was tested by intracutaneous

injection of 0.1 ml of tuberculin purified protein derivative (Statens Seruminstitut, Copenhagen, Denmark) and of 0.1 ml ovalbumin (300 µg/ml) two weeks after tertiary immunization. Monoarthritis (= AIA) was induced by injecting 1 ml (= 5 mg) ovalbumin into the knee joint. Injection of 1 ml sterile saline into the other knee was used as a control (= healthy control joint).

#### *Intra-articular treatments*

One group (n = 10) was subjected to weekly intra-articular injections into arthritic joint of 0.5 ml of liposomal clodronate (0.29 mg/ml) while another group (n = 7) received 0.5 ml of clodronate-free "empty" liposomes. Each rabbit received 7 injections. Sixteen hours after the last injection, the rabbits were sedated and sacrificed by i.v. Mebumat (50-80 mg/kg).

#### *Synovial and cartilage sample collection*

Patellas and synovial samples from intrapatellar, superolateral and posterior locations were collected. Patellas were decalcified and paraffin-embedded as described (18). Synovial samples were formalin fixed and paraffin embedded.

#### *Purification of COMP and production of antibodies*

Native COMP was purified from bovine tendon according to Hauser *et al.* (9) with minor modifications (19). After isolation using DEAE-Sepharose Fast Flow, Sepharose CL-4B and Mono Q 5/5 FPLC, thrombospondins were removed by affinity chromatography on heparin coupled to Sepharose CL-6B column. Fractions analyzed under reducing conditions in sodium dodecyl sulfate-polyacrylamide gel electrophoresis disclosed only one band of about 116 kDa. This band was blotted to polyvinylidene fluoride membranes. Its N-terminal sequence (Applied Biosystem gas phase sequencer) was Gly-Glu-Met-Pro-Leu-Gly-Gly-Asp, which is homologous to human COMP. Purified COMP was used as an antigen for immunization of New Zealand rabbits to raise a polyclonal anti-COMP antiserum. Immunoglobulin fraction

**Table I.** COMP positive cell proportion<sup>1</sup> (median) in different layers of the cartilage in healthy controls, in antigen induced arthritis treated with empty liposomes and in antigen induced arthritis treated with liposomal clodronate.

	The proportion of COMP immunoreactive cells
Superficial cartilage, controls	2
Superficial cartilage, treated with empty liposomes	0-1
Superficial cartilage, liposomal clodronate treated	2
Middle cartilage, controls	1
Middle cartilage, treated with empty liposomes	1
Middle cartilage, liposomal clodronate treated	2
Deep cartilage, controls	2
Deep cartilage, treated with empty liposomes	3-4
Deep cartilage, liposomal clodronate treated	3-4

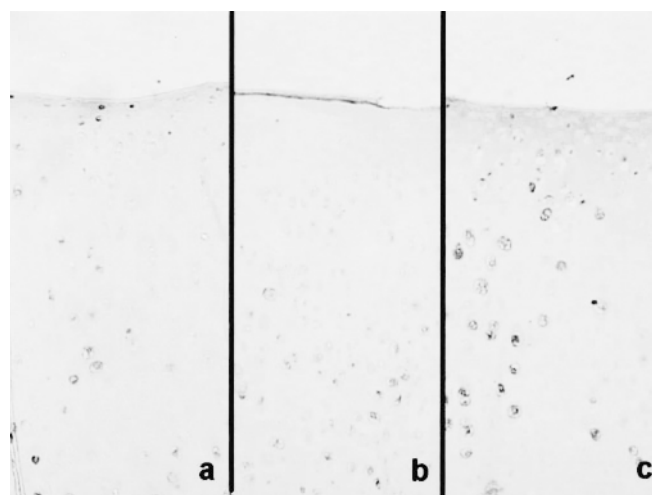
<sup>1</sup>The proportion of COMP positive cells was graded as follows: 0 = no positive cells, 1 = < 1 % positive cells, 2 = 1-25 % positive cells, 3 = 25-50 % positive cells, 4 = > 50 % positive cells).

was purified by precipitation and a slight cross-reactivity against fibronectin was removed by passing the antiserum through a fibronectin-Sepharose column.

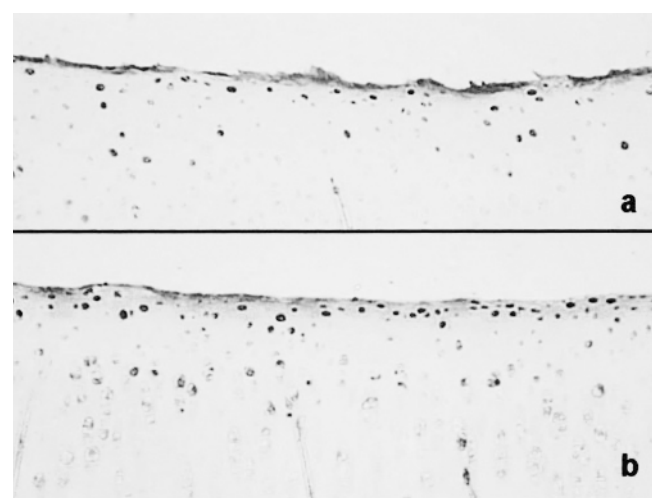
#### *COMP immunohistochemistry*

5 µm paraffin sections were heated for 10 minutes at 60°C followed by deparaffinization. Endogenous peroxidase was inhibited. Hidden antigens

**Fig. 1.** Micrographs of COMP immunohistochemistry comparing healthy control cartilage (a) to cartilage in antigen induced arthritis treated with intra-articularly administered empty liposomes (b) or liposomally packaged clodronate (c) (x 100).



**Fig. 2.** Micrographs of COMP stained cartilage sections showing the surface effect of liposomal clodronate in antigen induced arthritis. The normal healthy cartilage (a) and the diseased, but treated cartilage (b) are shown (x 100). Normal COMP expression pattern was preserved in the superficial layer of the cartilage.

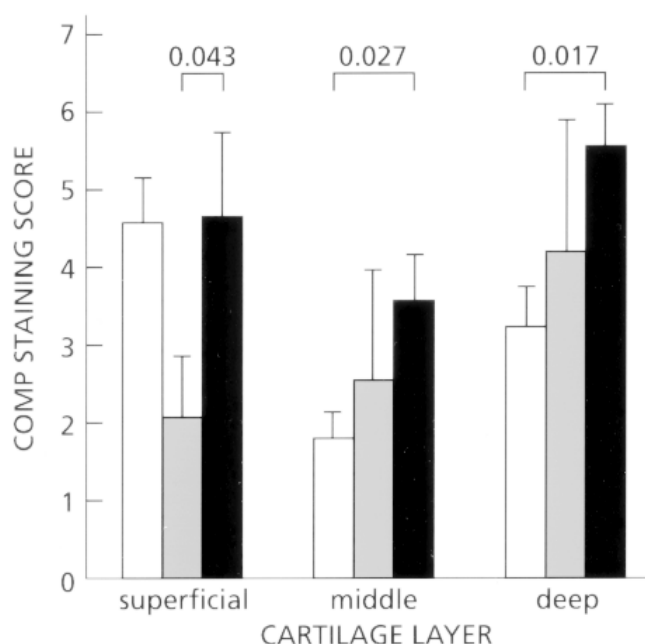


were revealed using a 30 minute digestion with 0.1% trypsin in 40 mU chondroitinase buffer. Block with 6% bovine serum albumin and 3% goat serum was used, followed by incubation overnight at 4°C with the 1:100 diluted primary rabbit anti-COMP antiserum. Control slides were treated with the corresponding nonimmune serum. Horseradish peroxidase labeled goat anti-rabbit IgG (Dako A/S, Glostrup, Denmark) was used as the secondary antibody for 1 hour at room temperature. Color was developed using H<sub>2</sub>O<sub>2</sub> and 3,3'-diaminobenzidine (Sigma). Sections were counterstained with Harris hematoxylin (Sigma), dehydrated and mounted in DPX.

### Evaluation

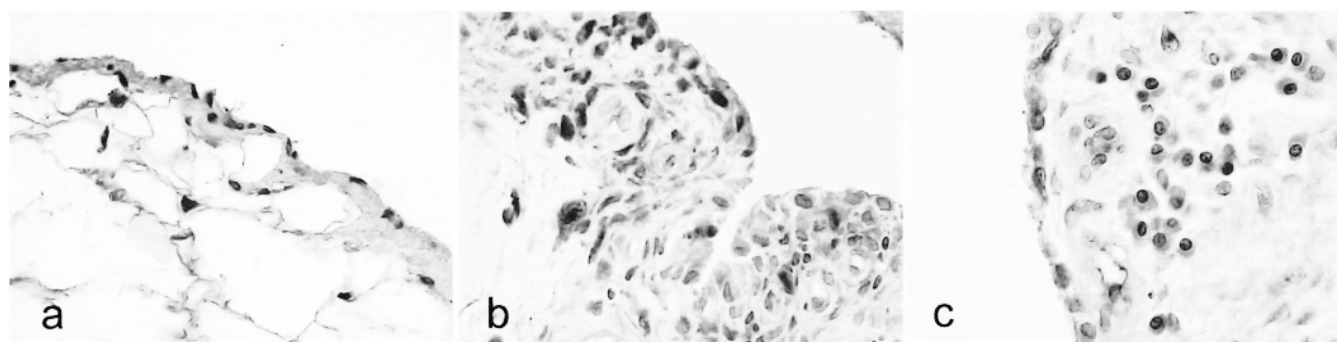
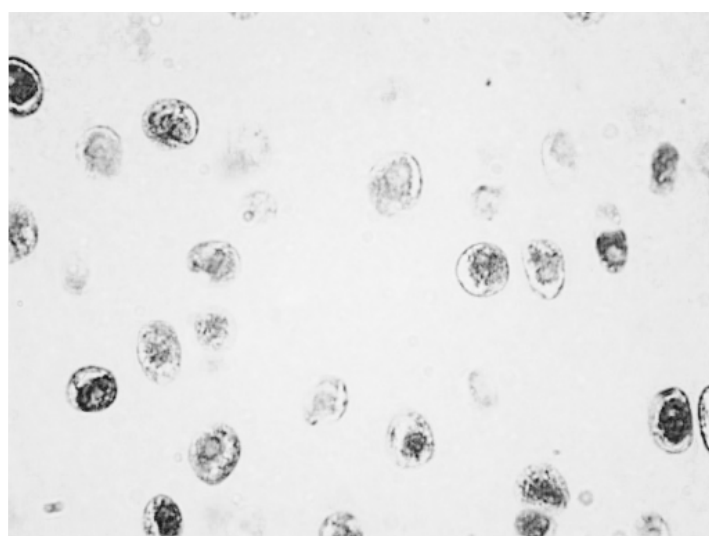
Cartilage sections were blindly evaluated for matrix and cellular COMP staining in cartilage areas far from capsular insertions, separately for the superficial (tangential), middle (transitional) and deep (radial) layers. Staining intensity of the extracellular matrix was graded as follows: 0 (none), 1 (weak), 2 (intense) and 3 (very intense), and evaluated in pericellular, territorial, and interterritorial matrix. The proportion of COMP positive cells was graded as follows: 0 (no positive cells), 1 (< 1% positive cells), 2 (1-25% positive cells), 3 (25-50% positive cells) and 4 (> 50% positive cells). In addition, cell density was recorded as follows: 0 (no cells), 1+ (scarce cells), 2++ (some cells) and 3+++ (many cells). Microphotographs were produced using a Nikon FDX-35 digital camera coupled to a Nikon Eclipse E400 microscope

**Fig. 3.** This graph displays the mean and the standard error of the mean of the cellular and matrix COMP staining of healthy contralateral controls (white bars), antigen induced arthritis treated with empty liposomes (gray bars) and antigen induced arthritis treated with liposomal clodronate (black bars). In the superficial cartilage layer, COMP staining was preserved in the liposomal clodronate treated arthritis group, whereas COMP staining was lost in the arthritis group treated with empty liposomes ( $p = 0.043$ , Wilcoxon test). Antigen induced



arthritis was associated with a compensatory increase of the cellular and matrix COMP in the middle and deep cartilage layers. This increase reached statistical significance between the healthy control joints and the contralateral liposomal clodronate treated arthritic joints ( $p = 0.027$  and  $0.017$  in the middle and deep cartilage layers, respectively, paired Mann-Whitney test).

**Fig. 4.** Cellular positivity for COMP as seen in the deep zone in liposomal clodronate treated cartilage (x 400).



**Fig. 5.** Micrographs of COMP staining of the synovial membrane in healthy control joints (a), in antigen induced arthritis treated with empty liposomes (b) and in antigen induced arthritis treated with liposomal clodronate (c) (x 400).

(Tokyo, Japan). Synovial membranes were evaluated separately for synovial lining and subsynovium.

### Statistics

Statistics was done using SPSS software (version 9.0; Chicago IL). Immunostaining scores are expressed as median and mean  $\pm$  standard error of the mean. Wilcoxon test was used to compare paired groups. Mann-Whitney test was used to compare independent variables. To improve the sensitivity for overall COMP changes in the tissue, matrix score values for pericellular, territorial, and interterritorial intensity staining were pooled together and considered as overall matrix staining. This was performed in the surface, middle and deep cartilage zones. A  $p < 0.05$  was considered significant.

## Results

### Cell density

The liposomal clodronate treated AIA cartilage was compared to AIA cartilage treated with empty liposomes and to healthy control cartilage. Chondrocyte densities did not differ ( $p > 0.05$ ) between these groups (liposomal clodronate treated AIA, empty liposome treated AIA and healthy control joints) in any of the analyzed cartilage layers (superficial, middle and deep layers) (data not shown). Table I reflects the results of chondrocyte COMP staining.

### Superficial cartilage layer

Liposomal clodronate treatment lead to preservation of the cellular architecture and matrix COMP, as shown in Figure 1, where a normal healthy joint, an AIA joint treated with empty liposomes and an AIA joint treated with liposomal clodronate are compared. COMP immunostaining of the matrix of the superficial layer, mostly interterritorial region, was seen in normal cartilage (Fig. 1, panel a, and Fig. 2, panel a). This staining was weak in arthritis treated with empty liposomes (Fig. 1, panel b), whereas COMP staining of the superficial matrix was well preserved and clear in liposomal clodronate treated joints (Fig. 1, panel c and Fig. 2, panel b).

The combined COMP staining score of

the interterritorial matrix and cells demonstrated preservation of COMP staining in the superficial layer in liposomal clodronate treated AIA joints, which was comparable to that seen in healthy control joints (Fig. 3). In contrast, there was a loss of COMP staining in the superficial layer of the cartilage in AIA joints treated with only empty liposomes compared to healthy controls or, more importantly, to arthritic liposomal clodronate treated joints (Fig. 3,  $p = 0.043$ ).

### Middle and deep cartilage layers

COMP staining of the middle and deep layers of the cartilage, an example shown in Figure 4, seemed to be intense and increased in all cases of AIA. This increase over the healthy control cartilage was slight and still non-significant ( $p > 0.05$ ) in AIA treated with empty liposomes, but reached statistical significance in the middle ( $p = 0.027$ ) and deep ( $p = 0.017$ ) cartilage layers in liposomal clodronate treated AIA (Fig. 3).

### Synovial membrane

In healthy control samples synovial lining was COMP positive (Fig. 5a). In addition, pericellular COMP staining was seen in subsynovium, mainly around fibroblasts. Furthermore, moderate COMP labeling was seen in the extracellular space in subsynovial connective tissue in healthy control joints. AIA treated with empty liposomes was characterized by lymphoid infiltrates and extensive fibrosis. Synovial lining was thickened and COMP positive (Fig. 5b). Pericellular labeling was seen around fibroblasts with intense and extensive interstitial COMP labeling of fibrous tissue in AIA compared to controls. COMP staining was also seen around macrophages and endothelia. No COMP labeling was detected in lymphoid aggregates. Liposomal clodronate treatment of AIA, due to its anti-inflammatory effect, has earlier been shown to moderate the synovial lining cell hyperplasia (7). Also according to the present results lining cells are diminished in numbers and are COMP positive (Fig. 5c).

## Discussion

The effects of high doses of clodronate

on AIA in synovium have been related to synovial lining type A macrophage destruction, as liposomal clodronate is internalized by synovial lining macrophages, which undergo apoptosis when subjected to high concentrations of clodronate (20, 21). Third generation bisphosphonates have been reported to suppress the severity of adjuvant arthritis in rats in a dose-dependent manner when used as either a prophylactic or therapeutic drug and administered subcutaneously (22). The present work extends these findings by demonstrating therapeutically useful effects upon direct intra-articular administration of clodronate. Clodronate is a non-amino bisphosphonate, which closely resembles pyrophosphate PPI and can therefore be metabolically incorporated into non-hydrolysable analogues of ATP that accumulate intracellularly resulting in apoptosis induction (23). In contrast, it has also been shown that low doses of clodronate, as used in the present study, do not induce apoptosis of synovial lining cells (7) and did not change the chondrocyte density in the hyaline articular cartilage as observed in the present study: all three layers of the cartilage had similar chondrocyte densities. Low, non-cytotoxic doses of clodronate exert anti-inflammatory properties both *in vitro* (24) and *in vivo* (7). This was confirmed also in the present study, which demonstrated increased COMP expression in synovitis in AIA, quite compatible with earlier findings demonstrating expression and cytokine mediated upregulation of COMP in synovitis (11, 25), and diminished COMP staining in inflammatory synovial membrane after intra-articular treatment of AIA with low-dose liposomal clodronate. It is possible that the present observations on the chondroprotective, structure maintaining effects of liposomal clodronate are in part secondary to the synovial anti-inflammatory effects. However, we consider it likely that liposomal clodronate also exert direct antiproteolytic and COMP protective effects (26-28). Furthermore, liposomal clodronate is also likely to be internalized by fibroblasts and chondrocytes as liposomes are used for *in vivo* gene transfer into

fibroblasts and chondrocytes. Intracellularly it may modulate gene transcription of matrix metalloproteinases (29) and, perhaps, COMP itself (see below). In any case, the present study demonstrates that cellular COMP behaves differently in different tissue compartments of the synovial joint. COMP acts as a marker of inflammation in synovial membrane, whereas it seems to indicate maintained structural integrity of the hyaline articular cartilage. Liposomal clodronate decreased COMP expression in synovial membrane, but maintained and/or enhanced it in the hyaline articular cartilage.

In human patients with rheumatoid arthritis and OA, COMP is released in increased amounts to synovial fluid and serum during the early phases of cartilage degeneration (30). These early reports were followed by others on the use of COMP as a marker of cartilage degradation or synovitis (14, 31). Fragments of the molecule were present in the diseased cartilage, synovial fluid and serum of patients with knee injuries, OA and rheumatoid arthritis (32, 33). In a population-based sample serum COMP levels distinguished unaffected comparators from OA, in which it reflected disease severity and involvement of multiple joints (34). We now extend these reports by demonstrating that intra-articularly administered low-dose, non-cytotoxic liposomal clodronate seems to exert chondroprotective effects. In AIA treated with liposomal clodronate COMP immunostaining was maintained or enhanced compared to AIA treated with only empty liposomes. COMP has important direct and indirect structure maintaining properties as its forms strengthening structural bridges between collagen type II and IX (10, 12) and a depot for cartilage growth/repair regulating hormones (16).

Usually, proteolytic solubilization and degradation have been considered to be responsible for the loss of COMP from cartilage matrix and its appearance in synovial fluid and blood plasma in arthritides. Also in the present study the most simple and straightforward explanation to the loss of COMP from the superficial hyaline articular cartilage in

AIA treated with empty liposomes (used as placebo) would be uninhibited proteolysis, whereas the protective effect of liposomal clodronate could be explained by prevention of it. However, it was in the present study clearly observed that the middle and low layers of the articular cartilage did not only "maintain" their COMP level but actually increased it. This is compatible with the observation by Salminen and coworkers (35), who reported on up-regulation of COMP mRNA and redistribution of the corresponding COMP protein during the early stages of OA in a transgenic mouse model with a dominant-negative mutation of type II collagen gene (36, 37). We conclude that deep cartilage in AIA does not undergo the COMP depletion observed in the superficial layer. This suggests an enhanced chondrocyte COMP production, probably as a result of the increased mechanical strain the damaged cartilage is subjected to (38-40). Liposomal clodronate seems to significantly enhance this protective host response so that COMP levels increase in the middle and deep cartilage layers. This may help to stabilize the collagen network structure and facilitates local deposition of small molecular weight molecules necessary for cartilage repair. This suggests a protective, anabolic effect of liposomal clodronate treatment on cartilage, which was not apparent when proteoglycans were used for the assessment of cartilage pathology (7). This extends the findings by Dickinson and coworkers (8), who noticed disease stage-dependent changes in proteoglycans, COMP and type II collagen, in this rank or process order. Our study demonstrates that also different layers of the hyaline articular cartilage respond differently to both disease and its treatment.

In conclusion, low-dose, non-cytotoxic liposomal clodronate exerts a dichotomous effect on synovial membrane and articular cartilage COMP in AIA model. This indicates that COMP is a useful marker for inflammation of the synovial tissue compartment and for the structural integrity of the hyaline articular cartilage. COMP is supposed to play an important role for the main-

tenance of the biomechanical properties and repair potential of cartilage as it helps to strengthen the collagen network and leads to local deposition of growth regulatory factors. As a result of locally delivered, intra-articular liposomal clodronate, COMP is maintained in the superficial cartilage layers and increased in the middle and deep cartilage layers, indicating that it is not only passively protected against proteolytic degradation and loss, but is also actively participating in the attempts to heal. As bisphosphonates are safe in long term use and inexpensive compared to biological host cytokine modulators (such as tumor necrosis factor modulators and interleukin-1 receptor antagonists), liposomally packaged bisphosphonates deserve more attention as potential chondroprotective agents.

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