Cartilage Destruction in Collagen Induced Arthritis Assessed with a New Biochemical Marker for Collagen Type II C-Telopeptide Fragments

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ABSTRACT. ***Objective.*** To assess the ability of a marker of collagen type II degradation (CTX-II) to quantify cartilage turnover *in vitro* in cartilage explants and *in vivo* in rats with collagen induced arthritis (CIA).

Methods. Bovine articular cartilage explants were cultured in the presence of interleukin 1α, oncostatin M, and plasminogen to induce cartilage degradation. CTX-II, CTX-I (C-telopeptide fragment of collagen type I), glycosaminoglycan, and hydroxyproline contents in culture supernatants were measured. CIA was induced in 12-week-old female Lewis rats by immunization with bovine type II collagen. The incidence and severity of arthritis were monitored by measuring paw swelling, and urinary levels of CTX-II and CTX-I were determined. The knee joints of rats were histopathologically examined after sacrifice.

Results. CTX-II but not CTX-I levels correlated well with collagen degradation in bovine articular cartilage *in vitro* quantified by hydroxyproline release. Urinary CTX-II levels as well as paw volume of CIA rats were significantly higher than normal rats on Days 21, 28, and 42 and were apparently correlated with cartilage destruction, assessed histopathologically. Urinary CTX-I level began to increase on Day 21, but only on Day 42 was it significantly different between CIA and normal rats. The elevation in CTX-I level appeared to occur later than that of CTX-II, in accord with the more delayed onset of bone erosion in the CIA model of rheumatoid arthritis.

Conclusion. Urinary CTX-II may be a useful marker for evaluation of dynamics of cartilage destruction in CIA rats. (J Rheumatol 2004;31:1174–9)

Key Indexing Terms:
BIOLOGICAL MARKERS COLLAGEN TYPE II CARTILAGE ARTHRITIS

Rheumatoid arthritis (RA) is a chronic inflammatory disease, characterized by hyperplastic synovial tissue, inflammatory infiltration, and progressive destruction of articular cartilage and subchondral bone. Articular cartilage is highly specialized tissue that covers the surface of synovial joints, allowing its smooth articulation. Cartilage is synthesized and maintained by chondrocytes, and is composed primarily of water, proteoglycan, and type II collagen. In both RA and osteoarthritis (OA) one of the major clinical manifestations is abnormal and degraded cartilage in the affected joint. Degradation and loss of articular cartilage are fundamental features of RA. However, in clinical practice, this variable is difficult to assess. Inflammation is evaluated by quantification of serum concentrations of acute phase proteins, and clinical symptoms are assessed using standardized rating methods for RA evaluation, such as American College of Rheumatology and Health Assessment Questionnaire scores. None of these methods provides a direct measure of joint destruction. To assess the progression of cartilage destruction, the most established method is the measurement of joint space width using plain radiographs, which provides a direct semiquantitative measure of bone erosion and destruction, but reflects the cartilage loss only indirectly. Although magnetic resonance imaging is more sensitive, standardized methods have not yet been established. Trained specialists and expensive apparatus are also required. Thus, a specific and sensitive biochemical marker of ongoing cartilage destruction in patients with joint disease is sought.

Several biomarkers have been used to identify processes leading to a defined clinical outcome in RA, such as joint space narrowing. Most of these markers are molecules released into biological fluid during the process of joint tissue turnover. Hedbom, *et al* reported cartilage oligomeric matrix protein (COMP) as a potential marker of cartilage

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turnover. COMP is a pentameric protein, originally purified from cartilage[1], but also shown to be present in other pressure loaded tissues, e.g., tendon and meniscus[2-4]. COMP can also be produced by cells in the dermal and synovial membrane[5]. YKL-40, also termed human cartilage glycoprotein 39, is a protein synthesized by chondrocytes and synoviocytes[6]. YKL-40 has also been shown to be increased in serum and synovial fluid of patients with RA. However, *in situ* hybridization studies with inflamed synovial tissue from patients with RA have demonstrated that YKL-40 is expressed in macrophage *in vivo*. YKL-40 is expressed *in vitro* by activated macrophages[7,8] and is exocytosed by activation from the specific granules of neutrophils[9]. Thus, COMP and YKL-40 lack specificity for cartilage degradation because of production from various tissues and cells except for cartilage. A number of markers are derived from collagen type II, which is the major component of cartilage[10]. Type II collagen is a specific product of cartilage[11]. The degradation products of type II collagen can be found in synovial fluid, serum, and urine, where they may be quantified to provide a measure of cartilage degradation[10,12,13]. Collagen type II propeptides may be measured in serum as anabolic markers of cartilage formation[10,14].

A new assay for measurement of collagen type II C-telopeptide fragments in urine (CartiLaps CTX-II, Nordic Bioscience, Herlev, Denmark) was recently developed; this marker is significantly elevated in patients with RA and OA[12]. Further, the CTX-II marker correlates to radiological measures of joint cartilage damage in both OA and RA[14,15], and elevated levels of the marker are associated with rapid progression of structural damage in OA and RA[14,16]. Thus clinical data suggest that CTX-II constitutes a specific marker of cartilage turnover in arthritis.

CTX-II has not been assessed in animal models of arthritis for its potential role as a biochemical marker of cartilage destruction. Animal models provide an essential tool for the study of pathological mechanisms and therapeutic interventions in inflammatory arthritis; the collagen induced arthritis (CIA) model is widely used as a model of RA. CIA is induced after immunization with cartilage derived type II collagen in mice, rats, or monkeys. Mice and rats with a permissive genetic background and specific MHC class II haplotype will develop CIA[17] with many similarities to human RA. Collagen type II reactive T and B cells are found in both CIA and RA[18,19], and the diseases show similar immunohistopathology, a chronic progressive disease course[20], and similar influence by sex hormones[21,22]. We examined the correlation between joint destruction in CIA rats and urinary CTX-II levels. The relationship between cartilage and bone destruction in the CIA model is discussed.

MATERIALS AND METHODS

Animals. Female Lewis rats (1-H2 haplotype) were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and bred in a clean atmosphere with 12 h light/dark cycles. Rats were fed standard rodent chow ad libitum and were free from infectious diseases. All experimental procedures were reviewed and approved by the Fujisawa Pharmaceutical Animal Experiment Committee.

Cartilage explant culture. Articular cartilage was obtained from adult cows and the cartilage was excised either as cylindrical plugs (5–30 mg) or as slices (20–30 mg). The explants were cultured in 96-well plates in 200 µl serum-free Dulbecco's modified Eagle's medium in the presence of recombinant human interleukin 1α (IL-1α), 5 ng/ml (Sigma, St. Louis, MO, USA), oncostatin M, 50 ng/ml (Sigma), and human plasminogen 10 µg/ml (Sigma). Plasminogen is a physiological activator of matrix metalloproteinases (MMP) required for degradation of collagen type II. The conditioned medium was harvested at various time points for measurement of biochemical markers.

Collagen induced arthritis. Bovine type II collagen (Collagen Research Center, Tokyo, Japan) was dissolved in 0.1 M acetic acid (2 mg/ml) and emulsified with an equal volume of incomplete Freund's adjuvant (Difco, Detroit, MI, USA). Twelve-week-old rats were immunized on Day 0 by intradermal injection on the back with 0.4 ml emulsion and at the base of the tail with 0.1 ml of the emulsion. On Days 7 and 14, rats were given an intradermal booster injection of 0.1 ml emulsion at the base of the tail. After immunization, the volume of the hindpaw was measured by a water displacement method using a plethysmometer for rats (MK-550; Muromachi Kikai Co., Ltd., Tokyo, Japan). Paw swelling was presented as a change in the hindpaw volume. Urine was collected as spot samples obtained by stimulating the abdomen of the rats. On Day 43, animals were sacrificed, and the right hind ankle joint was removed.

Histological evaluation. The right hind ankle joint of each rat was fixed in 10% neutral buffered formalin on Day 43. After decalcification, the joints were embedded in paraffin, sectioned longitudinally, stained with hematoxylin and eosin, and evaluated under light microscopy. Histological analysis was carried out on the basis of cartilage destruction. The severity of the lesions was classified into 4 grades: 0 = normal, 1 = mild change, 2 = moderate change, 3 = marked change.

Measurement of glycosaminoglycan (GAG) and hydroxyproline. The GAG proteoglycan content (as a measure of proteoglycan release) in the medium from cartilage explant cultures was measured by dimethylmethylene blue assay (Blyscan, Biocolor Ltd., Newtownabbey, Northern Ireland, UK) as described[23]. Hydroxyproline release (as a measure of collagen degradation) was assayed biochemically by modification of a described method[24].

Measurement of CTX-II. CTX-II was measured by a new competitive ELISA (CartiLaps; Nordic Bioscience) based on a mouse monoclonal antibody raised against the EKGPDP sequence of human type II collagen C-telopeptide, as described[12]. This sequence is found exclusively in type II collagen and not in other collagens, including type I collagen or other structural proteins. The antibody used in this assay is absolutely specific for peptides containing a free C-terminal proline[12]. The assay was performed by first incubating biotinylated EKGPDP peptide on a streptavidin microtiter plate and then sample as well as primary antibody was added. After overnight incubation, the plates were washed and a peroxidase labeled secondary antibody was added, followed by a chromogenic peroxidase substrate. The concentration of the CartiLaps ELISA [ng/l] was standardized to the total urine creatinine [mmol/l]. Measurement precision of the assay was 7.1% and 8.4% for intra and interassay variability, respectively.

Measurement of CTX-I. The RatLaps ELISA (Nordic Bioscience) was used to detect urinary excreted degradation products from the C-terminal telopeptide of type I collagen (CTX-I, a marker of bone turnover[25,26]). Urinary CTX-I measures were corrected for creatinine. Measurement precision of the assay was 6.9% and 10.6% for intra and interassay variability, respectively.

Statistical analysis. Results are expressed as the mean ± SEM. Significance

of differences between normal and arthritis groups was determined by nonparametric Wilcoxon rank-sum test or Student t test. Differences between groups were considered significant when the probability was less than 5% ($p < 0.05$).

RESULTS

Catabolism and release of CTX-II in bovine articular cartilage explants. In vitro culture of articular cartilage explants in the presence of the proinflammatory cytokines IL-1α, oncostatin M, and the MMP activator plasminogen induced degradation of the extracellular matrix. GAG gradually increased in the supernatant without any stimulation of catabolism. In the presence of IL-1α, oncostatin M, and plasminogen, GAG release was profoundly increased (Figure 1A). No more GAG was released after Day 10.

Hydroxyproline release in the conditioned medium was evident from around Day 10, and the concentrations of this metabolite reached a plateau on Day 20, indicating that no further release occurred after this time. The time course of release of CTX-II was quite similar to that of hydroxyproline (Figure 1B). Values of CTX-II and hydroxyproline were not detected in the supernatant without stimulation. We detected no CTX-I in the culture medium of cartilage explant cultures (data not shown). Thus, CTX-II levels correlated very well with the *in vitro* degradation of cartilage in cartilage explants.

Age-dependent decrease of urinary CTX-II in normal rats. The influence of rat age on urinary CTX-II levels was determined by collecting urine samples from normal female Lewis rats aged between 8 and 18 weeks. CTX-II and body weight of rats were measured at regular intervals throughout the observation period. These results are shown in Figure 2A and 2B. Body weight of Lewis rats increased gradually from age 8 weeks to 18 weeks. CTX-II levels in 12-week-old rats were substantially decreased compared to 8-week-old rats. After 12 weeks, CTX-II levels decreased more gradually during the next 6 weeks of the observation period. To minimize the age influence on CTX-II levels, we used rats aged 12 weeks for the following experiments to test the relationship between urinary CTX-II and development of arthritis.

CTX-I and CTX-II levels in CIA rats. In rats immunized with bovine type II collagen, the clinical inflammatory score measured as paw swelling was clearly manifested at Day 21 after the first immunization. Paw swelling persisted up to Day 42, when the experiment was terminated (Figure 3A).

The urinary CTX-II levels of normal rats decreased gradually and age-dependently from age 12 to 18 weeks, as shown in Figure 2A. The CTX-II levels of rats decreased transiently 7 days after the first immunization, and increased thereafter. The CTX-II levels in CIA rats were significantly higher than those of normal rats on Days 21, 28, and 42 (Figure 3B).

The individual levels of urinary CTX-II values and paw volumes were comparable in immunized and nonimmunized normal rats, whereas substantial variations were seen among the CIA rats (data not shown). One immunized rat did not develop CIA, which was apparent as absence of paw swelling, and CTX-II levels did not increase in this rat. In the rats that developed arthritis, CTX-II levels were elevated almost simultaneously with clinical onset of disease as monitored by the increase in paw volume. These results suggest that the change in CTX-II levels correlates with the change in paw swelling.

Urinary CTX-I levels in normal rats decreased slowly as the rats aged. The CTX-I levels of CIA rats showed no significant change from normal rats during the experimental period, until Day 35. However, on Day 42, CIA rats showed significantly higher levels of CTX-I compared with normal rats. Thus the increase in CTX-I was delayed for 2 or 3 weeks compared to CTX-II and paw volume (Figure 3C).

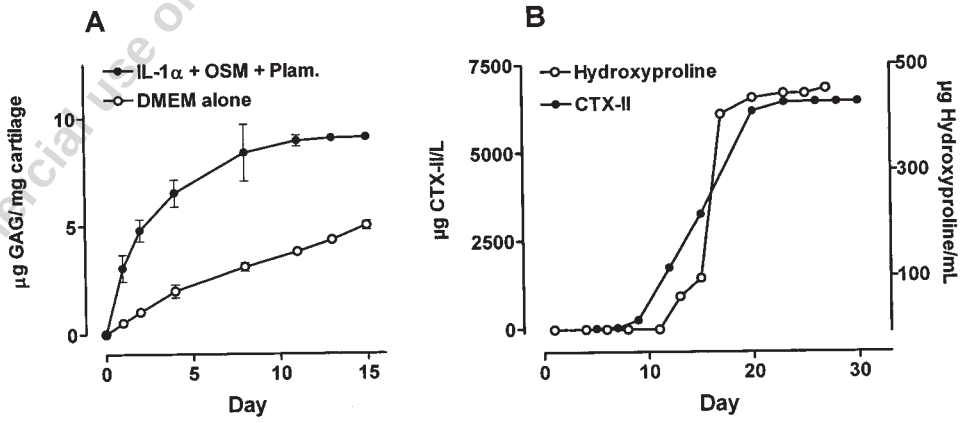

Figure 1. Release of glycosaminoglycan (GAG), hydroxyproline, and CTX-II from cartilage explant cultures. Cartilage explants were cultured in the presence of IL-1α, oncostatin M, and plasminogen for the indicated period of time. GAG (A), hydroxyproline, and CTX-II (B) were measured in the supernatants at regular intervals. Data are expressed as the mean of triplicate experiments.

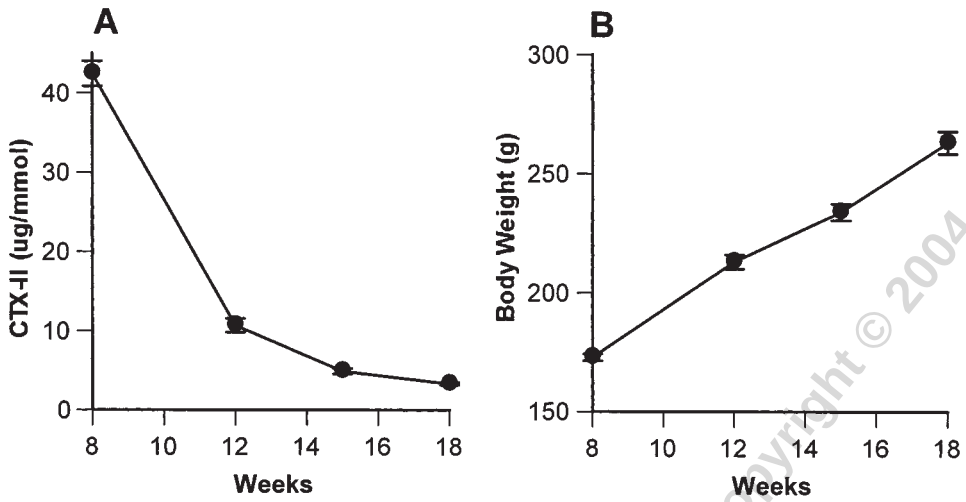

Figure 2. CTX-II and body weight changes with age in normal Lewis rats. Urine was collected from rats at 8, 12, 15, and 18 weeks of age; CTX-II values in urine were measured by ELISA and corrected for creatinine. CTX-II values (A) and body weights (B) were expressed as mean ± SEM of 5–10 animals.

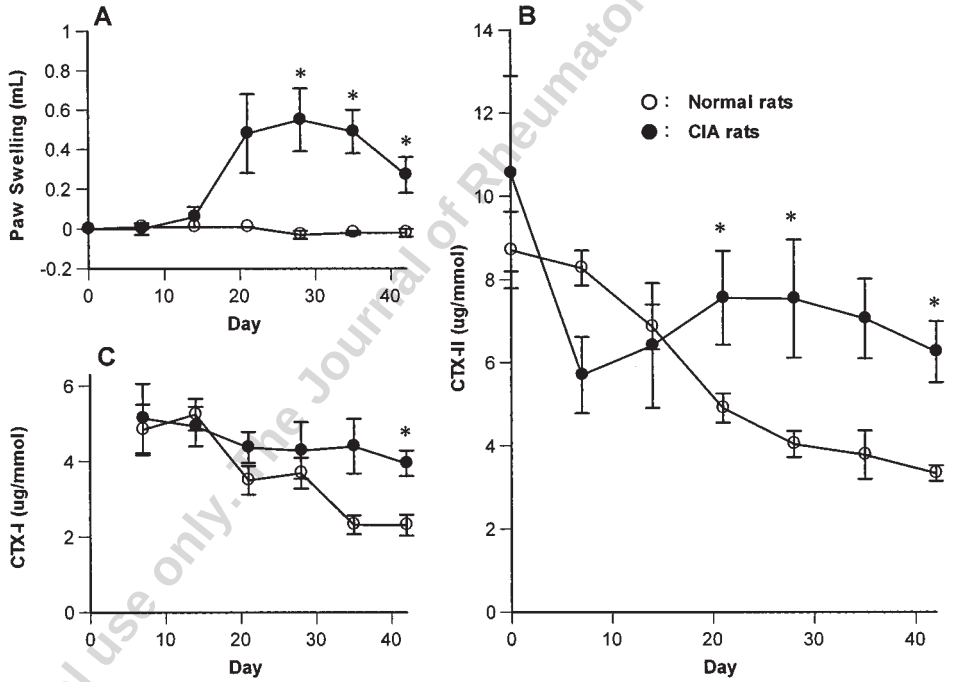

Figure 3. A. Paw volumes of normal and CIA rats. Rats were immunized with type II collagen at age 12 weeks. Paw volume was measured every 7 days from before the first immunization until 18 weeks of age. B. CTX-II values of normal and CIA rats. Urine was collected from rats every 7 days from before the first immunization and until 18 weeks of age. CTX-II values in urine were measured by ELISA and corrected for creatinine. C. CTX-I values of normal and CIA rats. CTX-I values in urine were measured by ELISA and corrected for creatinine. Results were expressed as mean ± SEM of 5 animals. *$p < 0.05$ compared with normal rats (Wilcoxon rank-sum test).

Relationship between cartilage destruction on histological examination and CTX-II. On the day after final urine collection, knee joints of normal and CIA rats were removed and evaluated by histological examination. There was marked joint destruction, with periarticular subcutaneous inflammatory edema and synovial effusion in the CIA group. Further, marked bone and cartilage destruction was observed in the CIA rats. Figure 4 shows the relationship between the histological score of cartilage destruction and the integrated levels of CTX-II from Day 21 to Day 42. The integrated CTX-II value as well as the cartilage destruction scores of CIA rats were significantly higher than in normal rats. There was good correlation between the CTX-II measures and cartilage destruction.

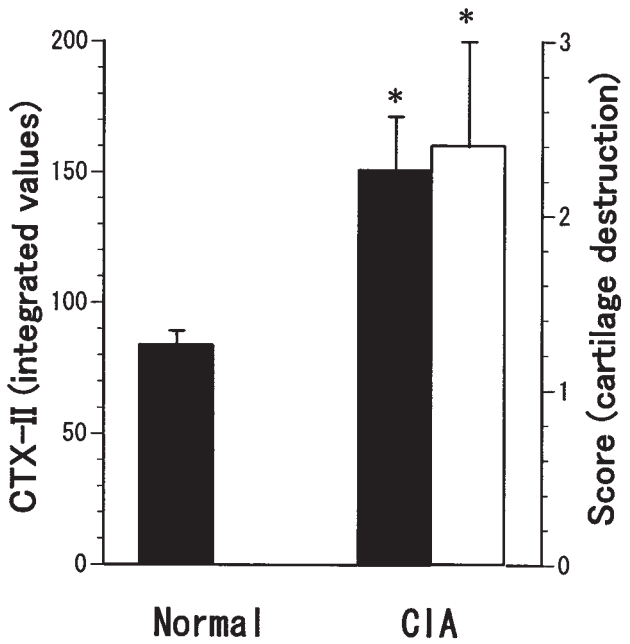

Figure 4. Correlation of histopathological examination of cartilage destruction with CTX-II. Black bars show mean levels of integrated CTX-II values from Day 21 to Day 42 in the CIA rats and the normal group. White bar shows the average histopathological score of cartilage destruction. Cartilage destruction score in normal rats was 0. Error bars represent SEM. *p < 0.05 compared with normal rats (CTX-II: Student t test; score: Wilcoxon rank-sum test).

DISCUSSION

In this study, metabolites of collagen type II were measured in culture supernatants of articular cartilage explants and in urine samples from the rat CIA model. Collagen type II metabolites were measured in a newly developed immunoassay specific for C-telopeptide fragments of the protein (CartiLaps CTX-II) in order to examine the correlation between cartilage destruction *in vitro* and *in vivo* and CTX-II. Type II collagen is the predominant collagen in cartilage and is restricted in localization to this tissue[11]. Clinical studies have shown that urinary levels of CTX-II are elevated in OA and RA, and that high CTX-II level is correlated with joint destruction and more aggressive disease[12,14-16].

At first, we assessed whether CTX-II release correlated with cartilage collagen degradation in the cartilage explant culture system. When cartilage pieces were cultured with IL-1α, oncostatin M, and plasminogen, the release of GAG from cartilage explants occurred rapidly after the culture was started (Figure 1A). However, CTX-II was only detected after 10 days of culture. Thus, the release of GAG significantly preceded collagen type II degradation, quantified by measurement of CTX-II (Figure 1B). The time course of CTX-II release was very similar to that of hydroxyproline release, strongly suggesting that the release of CTX-II directly indicates degradation of collagen in cartilage.

Next, we examined the correlation between urinary CTX-II levels and development of arthritis in CIA rats. Before the experiment, we confirmed the natural time course of systemic collagen type II turnover, as assessed with the CTX-II marker, from 8 to 18 weeks. In accord with the rapid growth seen in rats of this age, body weight increased almost 30% in this 10-week period (Figure 2). Urinary CTX-II decreased steeply from 8 to 12 weeks, whereas a slower decline was seen from 12 to 18 weeks (Figure 2). These dynamics of CTX-II measures suggest that a substantial amount of the collagen type II fragments measured in the assay are derived from turnover of the growth-plate, which contains collagen type II. In older animals the rate of skeletal growth is reduced, decreasing the contribution of growth-plate turnover to the CTX-II measures. This observation formed the rationale for using 12-week-old Lewis rats for the following experiments in order to minimize the age factor and the potential influence of growth-plate turnover on systemic concentrations of the CTX-II marker.

In the CIA rats, urinary CTX-II levels showed a pronounced decrease 7 days after the first immunization, and thereafter increased and remained higher than in normal rats (Figure 3). The nonimmunized healthy rats showed a steady decrease in CTX-II levels over the 42-day study period, in accord with the CTX-II levels measured in control rats (Figure 2). The time course of the remarkable increase in CTX-II coincided with that of the paw swelling. In clinical studies, high levels of CTX-II predicted the increased risk of progression of joint destruction in early RA in patients without radiologic evidence of joint damage[15,16]. Our results in CIA rats were in accord with the early detection and prediction of structural damage associated with RA in clinical studies. The CIA rats with high urinary CTX-II clearly showed destruction of the cartilage layer and bone erosion in histopathological examinations. Interestingly, one rat showed no paw swelling. In this animal, CTX-II remained at control levels and no histological sign of cartilage erosion was seen at study termination. Figure 4 shows that the integrated CTX-II levels observed in the study period when the arthritis was clinically expressed (Days 21 to 42) were significantly correlated with cartilage destruction detected by histological examination. These results strongly suggest that urinary CTX-II reflects articular cartilage destruction accompanying arthritis disease.

CTX-I, a collagen type I C-telopeptide fragment that is a marker of bone resorption[25,26], also gradually decreased in normal rats, but not in CIA rats. Urinary levels of CTX-I among CIA rats were significantly higher than in normal rats on Days 35 and 42. The dynamics of the CTX-I showed a different pattern than the CTX-II: significantly higher levels of CTX-II appeared from Day 21. Thus, the CTX-I release occurred later than CTX-II release. This is consistent with the process of joint destruction in patients with

arthritis, where the articular cartilage surface is destroyed by mechanical and/or enzymatic stress prior to destruction of the subchondral bone. The association between measures of bone and cartilage turnover in this model of inflammatory arthritis is not surprising, given the potential link between regulation of bone resorption and the inflammatory process in CIA. Osteoclast differentiation is stimulated by RANKL, which is expressed and secreted by activated T cells and fibroblasts in inflamed synovium. Further, synovial macrophage-like cells have been suggested to differentiate into osteoclasts in the inflamed joint in RA[27]. Both osteoclasts and chondrocytes express RANK and are thus able to respond to RANKL present in the environment. Bone and cartilage degradation could be under the control of the same activation pathway, since osteoprotegerin (endogenous decoy receptor counteracting RANKL) given *in vivo* to rats with adjuvant arthritis protects from bone and cartilage erosion, but not from inflammation[28].

Our study showed a possible correlation between urinary excretion of CTX-II and cartilage destruction in an arthritis model in rats. We also observed that bone resorption increases in arthritic rats, as indicated by the elevated CTX-I concentrations. CTX-II may be a good marker of cartilage destruction, which may provide a useful tool for monitoring the condition of arthritic disease.

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