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Early Elevation in Circulating Levels of C-Telopeptides of Type II Collagen Predicts Structural Damage in Articular Cartilage in the Rodent Model of Collagen-Induced Arthritis

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Objective. **To investigate changes in the circulating levels of the C-telopeptide of type II collagen (CTX-II) with relation to disease onset and structural damage of cartilage in a rodent model of collagen-induced arthritis (CIA), and to investigate immunolocalization of the CTX-II epitope in the articular cartilage of affected joints.**

Methods. **Seven-week-old female Lewis rats were immunized with type II collagen and monitored using blood sampling at weekly intervals. At study termination (day 23), the animals were killed, synovial fluid was collected, and the affected joints were scored macroscopically for disease severity and underwent immunohistochemical evaluation.**

Results. **At the time of disease onset (day 15), which was characterized by redness and swelling of the affected joints (mean ± SD macroscopic severity score 9.1 ± 1.6), there was a 355% increase in serum CTX-II levels. The early change in serum CTX-II from day 0 to day 15 showed a significant association with the severity of cartilage damage ($r = 0.61$, $P < 0.01$). Immunostaining revealed extensive presence of the CTX-II epitope in the damaged, uncalcified cartilage tissue.**

Conclusion. **The elevation in serum CTX-II concomitant with the onset of disease and proportional to cartilage damage demonstrates that CTX-II is a sensitive diagnostic tool for monitoring joint disease in the rodent model of CIA. Furthermore, the immunohistochemical findings are consistent with the concept that the major source of serum CTX-II is the damaged articular cartilage.**

Rheumatoid arthritis (RA) is a prototypic inflammatory joint disease that causes a severe burden for patients in terms of quality of life and functional abilities (1). Although much has been clarified regarding the pathophysiology of the disease, early detection of cartilage damage and effective chondroprotection remain considerable challenges for health professionals.

There are numerous animal models that have been instrumental in increasing our knowledge of the course and events of inflammatory joint diseases (2). One of the models of choice by which to study RA is the rat model of collagen-induced arthritis (CIA). Studies show that urinary hydroxylysylpyridinoline (HP), lysylpyridinoline (LP) (3), and cartilage oligomeric matrix protein (COMP) (4) provide useful noninvasive tools to monitor disease activity in the CIA model. However, due to limited specificity for articular cartilage, these biomarkers may not be the best with which to monitor the severity of cartilage damage in different phases of the disease or to assess the effect of different medical interventions in terms of chondroprotection.

Type II collagen (CII) is a major component of articular cartilage (5). We have recently introduced a novel assay to measure the concentration of C-telopeptides of type II collagen (CTX-II) in serum and synovial fluid samples, and demonstrated its utility to reflect structural alterations of articular cartilage in

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Dr. Chouinard, Ms Doyle, and Ms Smith own stock and/or hold stock options in Charles River Laboratories. Dr. Qvist owns stock in Nordic Bioscience.

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various experimental models of destructive joint disease (6). When widespread joint lesions were present (day 22), we found a significant correlation between serum/synovial fluid levels of CTX-II and the severity of cartilage damage. However, the utility of the CTX-II marker in a prospective setting has not yet been assessed.

Therefore, the present study was designed to investigate changes in the serum CTX-II level in relation to the onset of inflammation in the joints, how these early changes correlate with the severity of articular damage at the time of study termination, and whether articular cartilage is a significant contributor to the generation of CTX-II fragments (immunostaining). Since destructive joint diseases go beyond the boundaries of articular cartilage and affect bone as well, we also monitored the changes in levels of an established marker of bone resorption, CTX-I (7).

MATERIALS AND METHODS

CIA in rats. Twenty 7-week-old female Lewis (LEW/ScN/CrCrlBR) rats were obtained from Charles River Canada (St. Constant, Quebec, Canada) and kept at Charles River Laboratories Preclinical Services Montreal (Senneville, Quebec, Canada). The animals were housed 2–3 per cage in a room maintained at 22 ± 3°C with a 12-hour/12-hour light/dark cycle. Food (RPR 5L35; PMI Nutrition International, Henderson, CO) and water were provided ad libitum. The protocol was approved by the Institutional Animal Care and Use Committee and performed according to the guidelines of the US National Research Council and the Canadian Council on Animal Care, and in accordance with the US Food and Drug Administration Good Laboratory Practice Regulations.

The animals were injected once subcutaneously in the dorsal region with a total of 1 ml of porcine CII (2 mg/ml in 0.05*M* acetic acid; Chondrex, Redmond, WA) emulsified in Freund's incomplete adjuvant. The 1 ml of CII was divided among 10 injection sites (100 μl in each site). Control animals received an equal volume of saline.

A blood sample (0.4 ml) was collected from the jugular vein of all animals prior to immunization and on days 7, 15, 18, and 23 postimmunization. At study termination (day 23), animals were killed, and the maximum volume of blood was collected from the abdominal aorta. The blood was centrifuged at 2,700 revolutions per minute at room temperature for 20 minutes, and serum was collected, aliquoted, and kept at −20°C. Animals were denied food the night prior to blood sampling.

Synovial lavage was performed on both knees by injecting 0.2 ml saline into each stifle joint. The limbs were gently flexed, extended 10 times, and subsequently the fluid was collected, pooled for both knees, and stored at −20°C.

Biochemical assessment of bone and cartilage damage. Biochemical assessment of bone and cartilage damage and degradation was performed by measurement of CTX-I and CTX-II collagen, respectively. Briefly, serum CTX-I levels were determined by the RatLaps enzyme-linked immunosorbent assay (ELISA) (Nordic Bioscience, Herlev, Denmark) using rabbit antibodies raised against the amino acid sequence EKSQDGGR originating from rat CTX-I collagen. The intra- and interassay coefficients of variation (CVs) were 9.2% and 14.8%, respectively, and the range was 0–188 ng/ml. Levels of CTX-II in serum and synovial fluid samples were measured by the Serum Preclinical CartiLaps ELISA (Nordic Bioscience) using a mouse monoclonal antibody (F4601; Nordic Bioscience) that recognizes the amino acid sequence EKGPDP of the CTX-II collagen. The intra- and interassay CVs were 7.8% and 12.2%, respectively, and the measurement range was 0–270 pg/ml.

Macroscopic examination. The macroscopic severity score was focused on signs of inflammation, such as redness and swelling, which were graded on a semiquantitative scale from 0 to 5 (0 = normal; 1 = mild but definite redness of the ankle or wrist, or apparent redness limited to individual digits; 2 = slight swelling of the ankle or wrist, without swelling of individual digits; 3 = moderate swelling of the ankle or wrist; 4 = severe swelling of the entire paw, including digits; and 5 = maximally inflamed limb with involvement of multiple joints). Macroscopic evaluation of disease activity was focused on the radiocarpal, femorotibial, and tibiotarsal joints. The following was set as the criterion for defining disease onset: at least 2 consecutive days of slight swelling in at least 2 joints with 1 joint scored with a minimum grade of 2 and the other with a minimum grade of 1.

Microscopic examination of joint damage. At study termination, the right femorotibial, tarsal, and phalangeal joints were collected and retained in neutral buffered 10% formalin, and histologic evaluation of the femorotibial and tarsal joints was performed. Joints were decalcified in formic acid, dehydrated using grade alcohols, cleared with xylene, and embedded in paraffin. Two sections were cut; 1 was stained with Safranin O and the other with hematoxylin and eosin. The severity of joint damage was examined by microscopic assessment of cartilage damage (e.g., fibrillations/erosions/clefts, chondrocyte necrosis, and proteoglycan loss), subchondral bone resorption, osteophytes, and inflammation. The severity of each lesion was graded semiquantitatively on a scale from 0 to 5 (0 = no change; 1 = minimal; 2 = slight; 3 = moderate; 4 = severe; and 5 = massive). Subscores of the individual lesions were summarized in a total score. One investigator (LC), who was blinded with regard to treatment codes, scored the histologic sections. The microscopic scoring method was adopted from Bendele (8).

Immunolocalization of CTX-II epitopes in articular cartilage. The immunohistochemical evaluation of CTX-II epitopes was performed in 3 joints (femorotibial, tibiotarsal, and phalangeal), as previously described (9). Briefly, to detect degradation products of CII, we used the antibody F4601. Three consecutive sections were used for immunohistochemistry. One section was stained with toluidine blue, a second section was stained with anti–CTX-II antibodies, and a third section was used as a negative control. To demonstrate the binding specificity of antibodies, the negative control section was incubated with a mixture of primary antibody and a 50-fold higher molar concentration of CTX-II peptide.

Statistical analysis. Values are expressed as the mean ± SD, unless otherwise indicated. Differences between the mean CTX-I levels, CTX-II levels, and microscopic severity scores in different groups were compared using the Mann-Whitney U test. Correlations between CTX-II and microscopic severity scores were assessed using Spearman's rank correlation. P values less than 0.05 were considered significant.

RESULTS

Macroscopic evaluation of the disease. On day 15 after immunization, all animals in the CIA group fulfilled the criteria for disease onset, showing signs of inflammation characterized by a macroscopic severity score of 9.1 ± 1.6. At this time point, none of the animals from the control group showed signs of inflammation at any investigated joints.

Assessment of cartilage and bone turnover using CTX-II and CTX-I levels. The levels of cartilage degradation and bone resorption were monitored during the study period by measuring serum CTX-II and CTX-I levels, respectively. As has been demonstrated previously (10), the CTX-II level progressively decreased with the advancing age of the rat. To evaluate the changes in serum CTX-II and CTX-I induced by CIA without interference caused by aging, the levels of markers in the negative control group were assumed to be 100% at each time point. The levels of CTX-II and CTX-I are shown in Figure 1. At disease onset, observed 15 days after immunization, an elevation in serum CTX-II levels of 355% compared with that in control animals was registered ($P < 0.01$). The increase in circulating levels of CTX-II continued throughout the study period, peaking at study termination (day 23) at a 650% elevation compared with controls (Figure 1A). Bone tissue involvement was demonstrated biochemically by measurement of CTX-I. At study termination, but not before, CTX-I was significantly elevated compared with controls (165% of negative control) (Figure 1B). At the same time, the relative increases in CTX-II levels in serum and synovial fluid in rats with CIA compared with controls were 750% and 3,297%, respectively (Figure 2).

Evaluation of joint lesions and association between the total microscopic severity score of joint lesions and CTX-II. At study termination, the total microscopic severity score of joint damage in the rats with CIA was 25.8 ± 1.2, whereas evidence of joint destruction was completely absent in the control animals. Subscores reflecting subchondral bone resorption were 6.9 ± 0.1 (range 6–8) in diseased animals, whereas no signs of bone damage were found in the control group. Accordingly, the above-mentioned higher CTX-I levels in rats with CIA were associated with histologic evidence of subchondral bone resorption. In addition, there was a strong association between changes in serum CTX-II levels on day 15 (disease onset) and the total microscopic severity score at study termination ($r = 0.61$, $P < 0.01$).

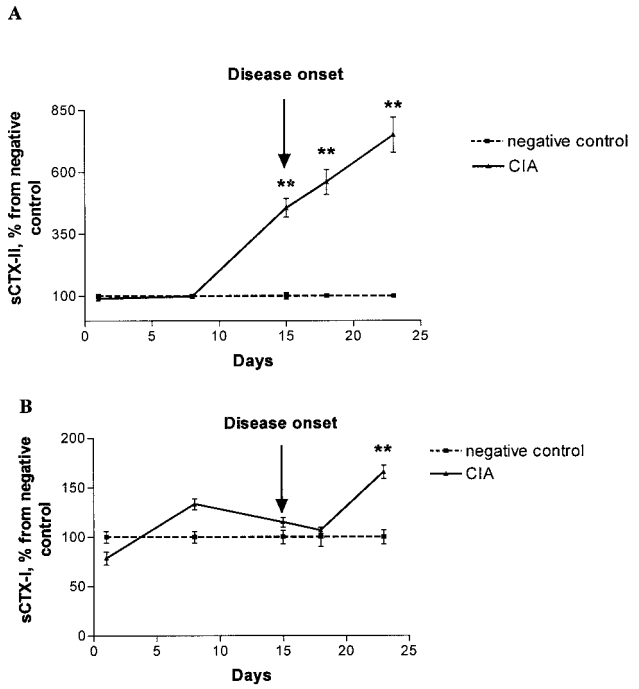

Figure 1. Turnover of **A,** cartilage and **B,** bone, assessed by measuring serum C-telopeptides of type II and type I collagen (sCTX-II and sCTX-I), respectively. Levels of the biomarkers in the collagen-induced arthritis (CIA) group were expressed as a percentage of those in controls (set at 100%). Values are the mean ± SEM. ** = $P < 0.01$ versus control.

Immunolocalization of CTX-II epitopes. To investigate whether CIA is accompanied by massive re-

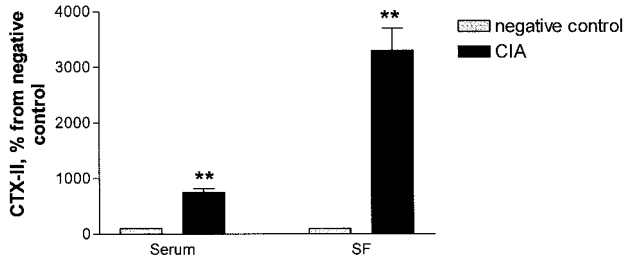

Figure 2. Levels of C-telopeptide of type II collagen (CTX-II) in serum and synovial fluid (SF) at the end of the study. Levels of CTX-II in the collagen-induced arthritis (CIA) group were expressed as a percentage of those in controls (set at 100%). Values are the mean and SEM. ** = $P < 0.01$ versus control.

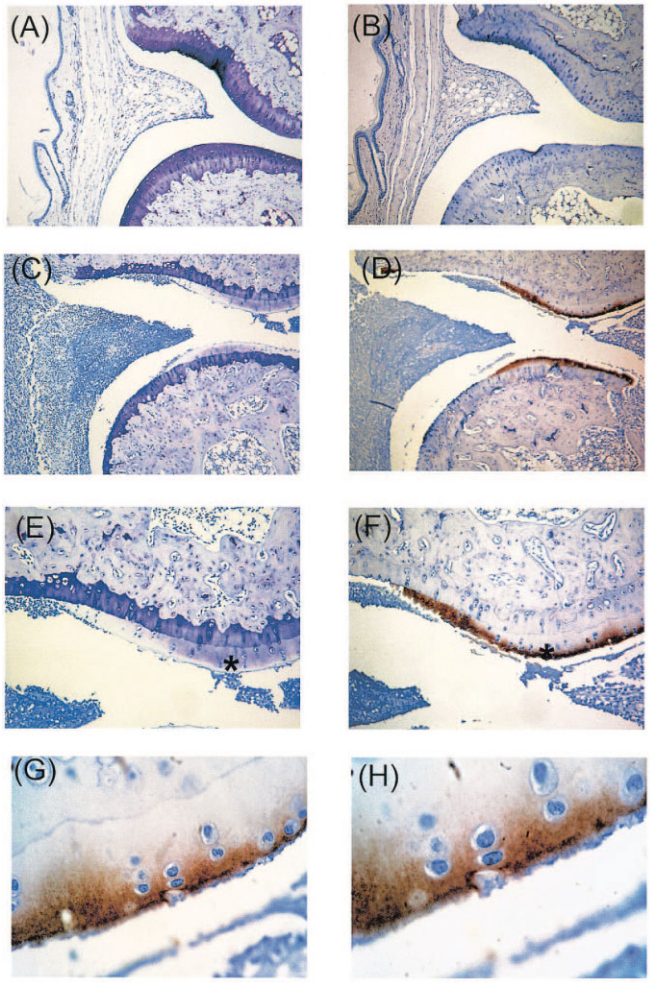

Figure 3. Immunostaining of C-telopeptide of type II collagen (CTX-II) epitopes associated with proteoglycan depletion of uncalcified cartilage in the phalangeal joint of a rat with collagen-induced arthritis (CIA) versus a control animal. **A** and **B**, Consecutive joint sections from a control animal, stained with toluidine blue and anti–CTX-II antibodies, respectively. **C** and **D,** Consecutive sections from a rat with CIA, stained with toluidine blue and anti–CTX-II antibodies, respectively. **E** and **F,** Areas of interest from **C** and **D**, at higher magnification. * indicates areas of colocalization of proteoglycan loss and CTX-II staining in articular cartilage. **G,** CTX-II staining in an animal with CIA. **H,** Area of interest from **G,** at higher magnification. (Original magnification × 10 in **A–D;** × 20 in **E** and **F;** × 60 in **G;** × 100 in **H.**)

lease of CII degradation fragments in articular cartilage, which would explain the marked increases in serum levels, we stained femorotibial, tarsal, and phalangeal joint sections from CIA and control animals using specific anti–CTX-II antibodies. Figure 3 shows representative findings in the phalangeal joint. The results provided evidence that the generation of CTX-II epitopes was colocalized with areas of marked proteoglycan loss (Figures 3E and F). No similar staining was revealed in the control animals (Figures 3A and B).

DISCUSSION

In the present study, we have shown that in rats with CIA, serum CTX-II was increased as early as the time of the first macroscopic clinical manifestation of the disease, and the early increase of serum CTX-II predicted the severity of structural alteration at the end of the study period. Immunostaining with anti–CTX-II antibodies revealed the extensive presence of CTX-II epitopes in articular cartilage of the affected joints.

Immunization of rats with CII induced a marked increase in the circulating level of CTX-II compared with control rats. These findings are consistent with those of previous studies of urinary CTX-II levels in rats with CIA (11). Furthermore, increases in CTX-II preceded the onset of disease and were significant at the time of occurrence of the first manifestations of joint inflammation. Previous studies showed significant elevations of C-reactive protein (CRP) before the onset of clinical disease (3), whereas other markers, such as alkaline phosphatase, HP, and LP, were elevated during the active phase of CIA (3). Thus, the diagnostic value of serum CTX-II in the early signaling of joint inflammation is similar to the value of measuring a systemic marker of inflammation (CRP).

Furthermore, the early increases in CTX-II showed good correlation with the severity of structural damage of articular cartilage at study termination. The predictive value of CII degradation markers for cartilage damage has been demonstrated in different models of destructive arthritis, such as osteoarthritis (OA) (10) and adjuvant-induced arthritis (12). The utility of CTX-II to predict disease progression of early RA in humans also has been demonstrated (13). Collectively, these findings indicate that CTX-II is not only an early indicator of joint inflammation in the CIA model, but also a reflection of progressive cartilage damage.

At the end of the study, serum CTX-I was also significantly elevated in the CIA group. The relative delay in the increase in CTX-I might suggest a secondary involvement of bone tissue. However, this is not the case. After synovial expansion and joint swelling, subchondral and marginal bone loss arise earlier in the course of the disease than does destruction of the articular cartilage. Basal levels of CTX-I are determined by the total skeleton (bone), which is much larger in volume than all of the articular cartilage. The increase in bone resorp-

tion in the subchondral and marginal regions of affected joints is probably too modest to significantly change systemic levels of CTX-I, and the disease must involve a larger portion of bone tissue before the increases can first be detected. The differences in the time course of increase in CTX-II and CTX-I levels are not driven by the sequence of events in the pathogenesis, but rather by the extent of collagen degradation in cartilage and bone in relation to the total volume of the respective tissue compartments.

Another important question was the source of CTX-II. In ovariectomized rats (9), we previously demonstrated mild CTX-II staining in damaged, uncalcified cartilage and the interface between bone and calcified cartilage. Compared with findings in the OA model, in the present study more pronounced and extensive CTX-II staining was found in the articular cartilage of all investigated joints (femorotibial, tarsal, and phalangeal) in the CIA group. These observations are similar to those reported by Stoop et al (14) in the murine antigen-induced arthritis model. The intensive immunostaining for CTX-II in the CIA model is due to a targeted immune response aiding in the elimination of CII.

At study termination, similar to previous findings (6), we found marked elevations of CTX-II in the synovial fluid of rats with CIA compared with controls. The magnitude of change exceeded that in the serum. This can be explained by a direct release of CTX-II fragments in the synovial fluid, which is in direct contact with surface erosions, whereas serum increases are a secondary phenomenon. Although the level of CTX-II in synovial fluid is useful for estimating the severity of cartilage damage in several animal models (6), its utility for monitoring purposes is limited.

In conclusion, we have demonstrated that measurement of serum CTX-II not only provides a useful estimate of disease activity but is also a predictor of cartilage damage in the CIA model. Based on these findings, it seems reasonable to assume that serum CTX-II can be a useful biomarker for monitoring the effects of chondroprotective drugs in the CIA model, a concept already corroborated by observations in humans (15).

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