Urinary Collagen Type II C-Telopeptide Fragments Are Sensitive Markers of Matrix Metalloproteinase-Dependent Cartilage Degradation in Rat Adjuvant-Induced Arthritis

FREDERIC DE CEUNINCK, MASSIMO SABATINI, VERONIQUE RENOUX, GUILLAUME de NANTEUIL, and PHILIPPE PASTOUREAU

ABSTRACT. ***Objective***. To assess the relevance of collagen type II C-telopeptide fragments (CTX-II) as markers of cartilage degradation during adjuvant-induced arthritis in rats.

Methods. Rats were injected with Freund's adjuvant on day 0 and treated orally for 21 days twice a day with vehicle or 10 or 20 mg/kg of a newly designed matrix metalloproteinase inhibitor (MMP-Inh). Urine samples were collected for 24 h between days 19 and 20 and the concentration of the cartilage-derived CTX-II was measured with a 2-site, sandwich-type ELISA. To assess arthritis, inflammatory scores were determined, and changes in paw volumes were measured by plethysmography.

Results. On day 21, the inflammation was generalized in rats injected with Freund's adjuvant. The urinary concentration of CTX-II was significantly higher in arthritic rats than in control non-injected rats. Oral treatment of arthritic rats with MMP-Inh dramatically decreased the concentration of CTX-II in urine, with values returning to those of controls. Treatment simultaneously reduced the clinical variables of the disease.

Conclusion. These results demonstrate that fragments of type II collagen in urine can be used as a measure of cartilage degradation in arthritic rats as well as potent non-invasive markers of the efficacy of chondroprotective treatments. (J Rheumatol 2003;30:1561–4)

Key Indexing Terms:
ADJUVANT-INDUCED ARTHRITIS TYPE II COLLAGEN BIOCHEMICALMARKERS URINE CARTILAGE DEGRADATION MATRIX METALLOPROTEINASE INHIBITOR

Although the biochemical processes of cartilage degradation in arthritic diseases are now better understood, there is a lack of routine assays that measure release of markers of these metabolic changes. In addition to their obvious usefulness in clinics, such circulating markers are much needed by pharmacologists to follow progression of disease in animal models and to study the efficacy of drug treatments in a non-invasive way.

To reflect the degradation of articular cartilage, markers should be very specific for this tissue. In addition, they should enter into the peripheral circulation through the draining lymphatics, and eventually into urine after renal filtration. These are 2 constraints that restrict the number of candidates. Serum cartilage oligomeric matrix protein (COMP) and urinary degradation products of type II collagen seem to be the most promising markers to date[1]. However, COMP is also produced by tendon, ligament, meniscus, and dermal or synovial fibroblasts[2,3]. Type II collagen is localized almost exclusively in cartilage, and its degradation by matrix metalloproteinases (MMP) is considered a critical event in the initiation and progression of arthritic diseases. Accordingly, various assays able to measure the degradation of type II collagen have been developed[4-6] and have proved to reflect joint damage when measured in biological fluids[5-7]. One of these assays that measures the C-terminal crosslinking telopeptide of type II collagen (CTX-II) in urine was proposed as a tool to monitor progression and/or therapy in osteoarthritis and rheumatoid arthritis in humans[1,6,8].

We investigated whether urinary collagen type II C-telopeptide fragments are sensitive markers of cartilage degradation in the rat model of adjuvant-induced arthritis, before and after treatment with a newly designed MMP inhibitor (MMP-Inh).

From the Departments of Rheumatology and Chemistry, Institut de Recherches Servier, Suresnes, France.

F. De Ceuninck, PhD, Scientist; M. Sabatini, PhD, Project Leader; V. Renoux, Technician; G. de Nanteuil, PhD, Director, Division of Chemistry; P. Pastoureau, PhD, Project Leader.

Address reprint requests to Dr. F. De Ceuninck, Division of Rheumatology, Institut de Recherches Servier, 11 rue des Moulineaux, 92150 Suresnes, France. E-mail: frederic.deceuninck@fr.netgrs.com

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MATERIALS AND METHODS

Inhibitory activity of MMP-Inh for MMP. The hydroxamate derivative

matrix metalloproteinase inhibitor hereinafter called "MMP-Inh," was synthesized at the Department of Chemistry of the Institut de Recherches Servier (IdRS). Synthesis and chemical characterization of this compound will be described elsewhere. The method used to determine the MMP inhibitory profile of MMP-Inh was that described in details by Chollet, *et al*[9]. Briefly, pro-MMP were activated by 2 mM p-aminophenylmercuric acetate and preincubated with various concentrations of the inhibitor for 30 min in 50 mM Tris-HCl containing 200 mM NaCl, $CaCl_2$ 5 mM, Brij 35 0.2%, pH 7.5. Then, the fluorogenic substrates (7-methoxycoumarine-4-yl)-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH2 (for MMP-3) or Dnp-Pro-Cha-Gly-Cys-(ME)-His-Ala-Lys(Nma)-NH2 (for MMP-1, -2, -9, and -13) were added to the mixtures for 6 h at 37°C. Substrate degradation in the presence or absence of MMP-Inh was measured by spectrofluorimetry, and the IC_{50} for each enzyme was calculated using the EXCEL software.

Assay for the degradation of type II collagen. Articular cartilage explants were obtained from the knee of New Zealand rabbits and cultured in 96 well plates at 2 explants/well in 120 µl of Dulbecco's minimal essential medium/Ham F12 medium (50/50) supplemented with 0.1% bovine serum albumin, 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies). Preliminary conditions were determined for stimulation of collagen degradation. Interleukin 1ß (IL-1ß) was used to induce production of pro-MMP, which were in turn activated by plasmin[10]. Plasmin was used instead of plasminogen in order to accelerate pro-MMPprocessing. Neither IL-1ß nor plasmin alone had any significant effect on collagen degradation, after 2 and 5 days of treatment. Only the combination of the 2 agents caused a strong stimulation of percent collagen release, which in a representative experiment increased, already by day 2, from 9% ± 1% in the control group to 50% ± 2% in the presence of IL-1ß plus plasmin [$p < 0.001$, by analysis of variance (ANOVA)]. According to these conditions, explants received vehicle alone (control) or treatment with 10 ng/ml IL-1ß and 0.1 U/ml plasmin plus vehicle. A dose-range of MMP-Inh in vehicle was added to the treated explants to determine IC_{50}. After a 48 h period, both explants and media were collected, and the hydroxyproline (OH-Pro) content in each fraction was determined after hydrolysis with 18.5% HCl and heating at 110°C for 12 h, followed by colorimetric detection as described[11]. The percentage of collagen degradation was calculated as:

OH-Pro content in medium/total OH-Pro (explant + medium) × 100

Results were calculated from 8 replicates for each condition. The IC_{50} for degradation of type II collagen was calculated from combined data of 2 experiments using EXCELsoftware.

Induction of adjuvant-arthritis and measurement of clinical inflammatory variables. Animals were housed in groups of 4 or 5 in large cages with water and standard diet ad libitum one week before the beginning of experimentation. Adjuvant-arthritis was induced at day 0 as described[12] by subcutaneous injection of Freund's complete adjuvant containing heat-killed *Mycobacterium butyricum* in the subplantar region of the right hind paw of female Lewis rats 63 days old. Animals were weighed every 2 days. Oral treatment with MMP-Inh was started just after injection of adjuvant and was administered twice a day at 10 or 20 mg/kg through a feeding tube. Each dose was corrected for body weight. Clinical assessment of the disease was determined at day 21 by measuring the increase of the left hind paw volume by plethysmography and by scoring inflammatory variables exactly as described[12], taking into account swelling, deformity, and ankylosis of non-injected paws, the redness and swelling of ears and the presence of nodules in ears and in the tail (maximum score per animal = 44). Two independent studies were carried out. The first study used a larger cohort of animals in order to study the CTX-II concentrations in control (n = 17) and arthritic (n = 18) rats. In the second study, CTX-II concentrations were measured in controls and MMP-Inh-treated animals (n = 7 to 8 per group). Ethical guidelines for experimental investigations in animals were followed[13]. The experimental protocol was used after consultation of the IdRS ethics committee. Every effort was made to minimize pain in the animals.

ELISA for CTX-II. The concentrations of CTX-II were measured blind in urine samples collected for 24 h between days 19 and 20. For this, rats were placed individually in metabolic cages in which urine was separated from feces before being collected in tubes. The competitive assay for CTX-II (Nordic Biosciences, Copenhagen, Denmark) has been described[6]. The lower detection limit was 0.3 µg/l. All samples were assayed in duplicate with coefficients of variation ranging between 0 and 12.7% (mean 4.1%). For each sample, the creatinine concentration was determined in duplicates to correct for variations in urine flow-rates.

Statistical analyses. Data are expressed as means ± standard error to the mean (SEM) for each group. For each variable, the homogeneity of variances was studied by F test and the significance of differences between arthritic and control groups was studied by the unpaired Student's t test (for significantly homogeneous variances) or the Satterthwaite test (for significantly heterogeneous variances). The effect of treatment with MMP-Inh was studied by an ANOVA followed by a Dunnett's t test, or a Kruskal-Wallis test followed by a nonparametric multiple comparison procedure for significantly heterogeneous variances[14].

RESULTS

MMP inhibitory profile of MMP-Inh. MMP-Inh exhibited inhibitory activity in the nanomolar range towards all MMP tested (Table 1). A somewhat lower selectivity was found for MMP-1, with a MMP-13/MMP-1 ratio of 33.3. In an *in vitro* assay for collagen degradation, using interleukin 1ß as a pro-MMP inducing factor and plasmin as an activator[10], MMP-Inh inhibited the degradation of type II collagen with an IC_{50} of 56 nM. Collagen degradation was inhibited by 83% at the concentration of 100 nM.

Measurement of the urinary CTX-II concentration in control and arthritic rats. The urinary concentration of CTX-II was measured in 17 control rats and 18 arthritic rats between days 19 and 20 (Figure 1). The CTX-II concentration was 26.7% higher in the arthritic group than in controls ($p < 0.001$). Values ranged between 9.2 and 14.1 µg/mmol creatinine in control rats (12.37 ± 0.30) and between 9.2 and 22.3 µg/mmol creatinine in arthritic rats (15.67 ± 0.84). In the arthritic group, 15 out of 18 rats had a CTX-II concentration above the mean control value, of which 12 were above the upper control value.

Relation between disease activity and urinary CTX-II concentrations after treatment with MMP-Inh. Body weight, paw edema, and inflammatory scores were determined on day 21 in controls (group A), arthritic rats (group B), and arthritic rats treated with MMP-Inh twice a day at 10 or 20

Table 1. Inhibitory profile of MMP-Inh on MMP activities and collagen degradation *in vitro.*

	IC_{50} (nM)
MMP-1	28
MMP-2	0.66
MMP-3	0.56
MMP-8	0.27
MMP-9	0.97
MMP-13	0.84
Collagen degradation	56

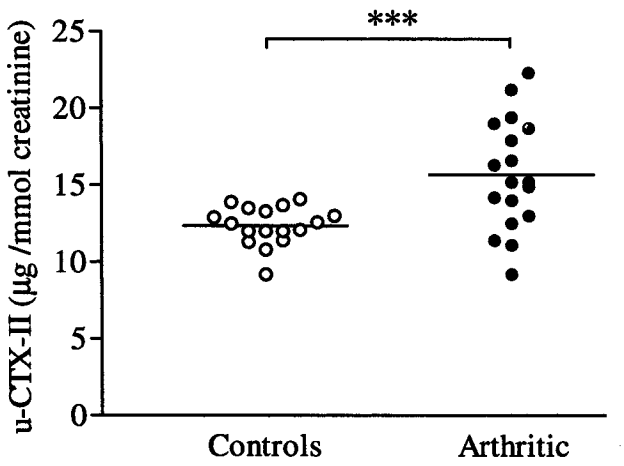

Figure 1. Urinary concentrations of CTX-II in age-matched control and arthritic rats. Individual measurements are corrected for creatinine content. Horizontal bars indicate the mean concentrations in each group. The statistical difference between the 2 groups was assessed by Satterthwaite t test: ***$p < 0.001$.

mg/kg (groups C and D) (Table 2). A 20.1% loss of body weight ($p < 0.001$) was observed in group B versus group A 21 days after injection of adjuvant. This loss was partially but significantly recovered in groups treated with MMP-Inh. The volume of left paw edema was dramatically increased in arthritic rats (1.28 ml vs 0.02 ml in controls; $p < 0.001$) and attenuated after treatment with MMP-Inh (–50.0% in group C, not significant; –71.1% in group D, $p < 0.05$) thereby demonstrating efficacy of the drug on progression of the disease. The inflammatory score in group B reflected the severity of the disease. This score was attenuated by about 25% in the 2 groups treated with MMP-Inh, without reaching statistical significance. The concentration of CTX-II in urine collected between days 19 and 20 was measured in each group (Figure 2). The CTX-II concentration was 11.87 ± 0.62 µg/mmol creatinine in group A and 16.30 ± 1.30 µg/mmol creatinine in group B (+37.3%, $p < 0.05$). At each dose, MMP-Inh restored CTX-II concentrations equivalent to those of controls (group C, 9.28 ± 0.50; group D, 9.50 ± 1.03; statistical difference between groups C or D and group B, $p < 0.001$).

DISCUSSION

In this inflammatory model characterized by a strong breakdown of cartilage, the marker studied is highly specific for cartilage and thus should reflect degradation of this tissue during progression of the disease. We found that the signs of the disease in arthritic rats, i.e., a decrease of the body weight, and a strong increase of the volume of paw edema and inflammatory scores, were accompanied by an increase of the type II collagen C-telopeptide fragments in urine. Thus, the urinary concentration of these fragments reflected the consequence of inflammatory events on cartilage degradation. Although no longitudinal measurements were made in this study, this observation is valuable and promising since it may avoid the use of invasive tools or complicated methods to assess cartilage breakdown in this pathology. Further, urinary CTX-II seemed to be sensitive enough to discriminate between a normal and an arthritic group, even with a relatively low number of animals.

It has been shown in earlier studies that MMP inhibitors that can prevent cartilage degradation *in vitro* are also capable of attenuating the clinical signs of adjuvant-arthritis in rats[15,16]. We were able to demonstrate that a new MMP inhibitor that decreased the degradation of type II collagen *in vitro* with high potency, not only decreased the clinical signs of the pathology, but also permitted a strong decrease of the urinary levels of CTX-II in arthritic rats, with values returning to those of the non-injected controls. We postulate that this model could be used for the identification of drugs that inhibit cartilage destruction *in vivo* and above all, that the measurement of degradation fragments of type II collagen could also be used as sensitive indicators of the efficacy of these drugs. In 2 clinical studies, urinary CTX-II levels were recently found to be responsive to anti-osteoporotic treatments in humans, thereby demonstrating dual activities of these drugs, both on bone tissue as assessed by measurement of the urinary excretion of type I collagen C-telopeptides (CTX-I), and on articular cartilage[17,18], as also independently demonstrated in a rabbit model of inflammatory arthritis[19].

In addition to the indirect demonstration that MMP play a part in the cleavage of type II collagen C-telopeptides in

Table 2. Body weights, paw edema, and inflammatory scores on day 21 of control rats, arthritic rats, and rats treated with MMP-Inh. Data are expressed as means ± SEM. Statistical differences in group B were calculated by comparison with group A by Satterthwaite test. Statistical differences in groups C and D were calculated by comparison with group B by the Kruskal-Wallis test followed by a nonparametric multiple comparison procedure.

Group	Body Weight (g)	Left Paw Edema (ml)	Inflammatory Score
A (control, n = 7)	207.4 ± 3.3	0.02 ± 0.01	0
B (arthritic, n = 8)	165.7 ± 1.1[c]	1.28 ± 0.22[c]	17.9 ± 2.8
C (treated 2 × 10 mg/kg, n = 8)	177.6 ± 3.4[a]	0.64 ± 0.18	13.0 ± 3.0
D (treated 2 × 20 mg/kg, n = 8)	178.8 ± 2.7[b]	0.37 ± 0.08[a]	13.4 ± 3.5

[a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$.

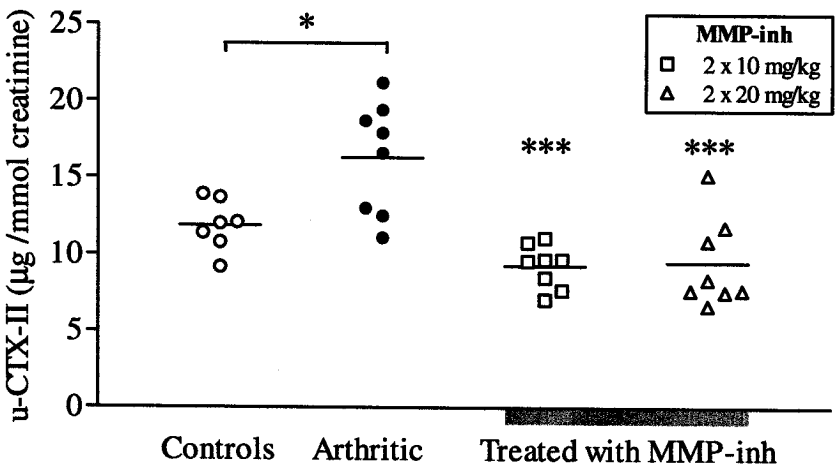

Figure 2. Urinary concentrations of CTX-II in control rats, arthritic rats, and rats treated with MMP-Inh. Individual measurements are corrected for creatinine content. The horizontal bars indicate mean concentrations in each group. Statistical differences between all groups were assessed by Dunnett's t test. Difference between control and arthritic rats: *$p < 0.05$. Statistical difference between arthritic rats and each treated group: ***$p < 0.001$.

cartilage, which was an unaddressed issue[6], our study primarily demonstrates that the urinary marker CTX-II may be considered as a promising pharmacological tool to evaluate the efficacy of drug treatments in animal models of joint pathologies.

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