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     BIOCHEMICAL MARKERS
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. However, COMP is also produced by tendon, ligament, meniscus, and dermal or synovial fibroblasts. 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 and have proved to reflect joint damage when measured in biological fluids. 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.

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).

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. 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-Cl containing 200 mM NaCl, CaCl₂, 5 mM, Brij 35 0.2%, pH 7.5. Then, the fluorogenic substrates (7-methoxyxocoumarin-4-y1)-Arg-Pro-Lys-Pro-Arg-Pro-Lys-Nva-Arg-Lys-Met (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 degrada-
tion in the presence or absence of MMP-Inh was measured by spectrofluorimetry, and the IC₅₀ 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. Adjuvant-arthritis was induced at day 0 as described. Each dose was corrected for body weight. Clinical assessment of the paw volume by plethysmography and by scoring inflammatory variables. Ethical guidelines for experimental investigations in animals were followed.

**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 18 as a pro-MMP inducing factor and plasmin as an activator, MMP-Inh inhibited the degradation of type II collagen with an IC₅₀ 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 22.3 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>
<thead>
<tr>
<th>IC₅₀ (nM)</th>
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<tbody>
<tr>
<td>MMP-1</td>
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<td>MMP-2</td>
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<td>MMP-9</td>
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<tr>
<td>MMP-13</td>
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<tr>
<td>Collagen degradation</td>
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Table 1. Inhibitory profile of MMP-Inh on MMP activities and collagen degradation in vitro.
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. 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, as also independently demonstrated in a rabbit model of inflammatory arthritis.

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

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**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.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (g)</th>
<th>Left Paw Edema (ml)</th>
<th>Inflammatory Score</th>
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<tbody>
<tr>
<td>A (control, n = 7)</td>
<td>207.4 ± 3.3</td>
<td>0.02 ± 0.01</td>
<td>0</td>
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<tr>
<td>B (arthritic, n = 8)</td>
<td>165.7 ± 1.1</td>
<td>1.28 ± 0.22</td>
<td>17.9 ± 2.8</td>
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<tr>
<td>C (treated 2 × 10 mg/kg, n = 8)</td>
<td>177.6 ± 3.4</td>
<td>0.64 ± 0.18</td>
<td>13.0 ± 3.0</td>
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<tr>
<td>D (treated 2 × 20 mg/kg, n = 8)</td>
<td>178.8 ± 2.7</td>
<td>0.37 ± 0.08</td>
<td>13.4 ± 3.5</td>
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* a p < 0.05; b p < 0.01; c p < 0.001.
cartilage, which was an unaddressed issue, 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.

REFERENCES