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The utility of measuring C-terminal telopeptides of collagen type II (CTX-II) in serum and synovial fluid samples for estimation of articular cartilage status in experimental models of destructive joint diseases

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Summary

Objective: To characterize and validate a novel enzyme-linked immunoassay for measuring cross-linked dimer forms of C-terminal telopeptides of type II collagen (CTX-II) in serum and synovial fluid of rodents, and investigate whether CTX-II measurements can reflect joint status in two established animal models of destructive joint diseases.

Methods: Firstly, the specificity, *in vivo* validity, antigen recovery, and reproducibility of the assay were investigated. Secondly, we induced arthritis in rats using either bovine collagen type II or mono-iodoacetate. CTX-II levels were measured in the serum and synovial fluid of the affected femoro-tibial joint and correlated with microscopic severity of joint lesions as determined by validated scoring systems.

Results: The F4601 monoclonal antibody (mAb) is highly specific for the EKGPDP sequence at the CTX-II. Strong CTX-II signals were detected during enzymatic degradation of articular cartilage explants by matrix metalloproteinase (MMP)-9 or MMP-13. The assay presented a good degree of precision and reproducibility (inter- and intra-assay coefficient of variations $< 8.0\%$). In the collagen-induced arthritis (CIA) model, the assay indicated markedly increased levels of CTX-II in both the synovial fluid and the serum. Furthermore, CTX-II levels in both the synovial fluid ($r = 0.76$; $P < 0.0001$) and the serum ($r = 0.85$; $P < 0.0001$) showed strong correlations with the microscopic severity scores of joint lesions at Day 22. In the mono-iodoacetate-induced arthritis (MIA) model, CTX-II concentration in the synovial fluid ($r = 0.53$; $P < 0.0001$), but not in the serum, correlated with the microscopic severity score.

Conclusions: The Preclinical CTX-II assay could provide a useful supplement to currently available methods for the non-invasive assessment of cartilage status. The utility of serum CTX-II to reflect joint status appeared to be limited to systemic forms of destructive joint diseases.

Key words: Collagen-induced arthritis, C-telopeptide of collagen type II, Microscopy, Mono-iodoacetate-induced arthritis, Rats.

Abbreviations: CIA type II collagen-induced arthritis, MIA mono-iodoacetate-induced arthritis, CTX-II collagen type II C-telopeptide degradation fragment, OA osteoarthritis, RA rheumatoid arthritis, COMP cartilage oligomeric matrix protein, MMP matrix metalloproteinase, CV coefficient of variation.

Introduction

Currently, there are numerous animal models available for preclinical research aiming for the better understanding of the pathophysiology, diagnostics, and treatment options of destructive joint diseases, such as rheumatoid arthritis (RA) and osteoarthritis (OA). Due to similar pathology and the presence of collagen type II-reactive T and B lymphocytes, the rat model of collagen-induced arthritis (CIA) is one of the most widely used models of RA[1–4]. OA can be induced by diverse ways, such as anterior cruciate ligament transection[5,6], ovariectomy[7], or intra-articular injection of mono-iodoacetate[8–10].

Currently, to assess the impact of novel disease-modifying agents on arthritis and related structural alterations of affected joints, we need to sacrifice the experimental animals and directly grade the microscopic structural changes in the affected joint. Non-invasive techniques reliably reflecting structural changes of articular cartilage remain sparse. Measurement of different degradation products of the cartilage matrix in synovial fluid may solve some of these problems, yet this approach is not ideal for monitoring purposes due to limited sample volume and major difficulties with obtaining serial samples. Therefore, there is a need for biochemical markers that are detectable in the serum and could ease the aforementioned technical difficulties for researchers.

In the past years, numerous biochemical markers have been developed and tested for their ability to serve such purposes. Of the different marker candidates, the cartilage oligomeric matrix protein (COMP)[11,12] and the C-terminal telopeptide of collagen type II (CTX-II)[4] have yielded

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promising results in animal models of RA. Furthermore, studies using the meniscectomy-induced OA model have demonstrated a significant correlation between CTX-II concentration in the synovial fluid (estimated by variant assay, Col2CTx) and the severity of OA graded by histological evaluation of articular cartilage[13,14]. The potentials of CTX-II are also corroborated by observations indicating that early changes in urinary excretion of CTX-II in ovariectomized rats show a significant correlation with subsequent structural changes of articular cartilage[7,15]. These experimental observations are corroborated by clinical findings indicating that urinary CTX-II is elevated in patients with RA and OA compared with healthy individuals, and levels of the biomarker are predictive for the rate of cartilage degradation[16–21]. Serial measurements of CTX-II can be helpful in monitoring the effect of potential disease-modifying drugs[22,23].

Herein we introduce a novel immunoassay, which targets cross-linked forms of CTX-II and allows measurements in both synovial fluid and serum samples from rodents. In addition to the standard technical validation of the assay, we demonstrate the utility of CTX-II measurements for reflecting structural alterations of articular joints in two well-established models of RA and OA, namely, the CIA and the mono-iodoacetate-induced arthritis (MIA), respectively.

Materials and methods

PART 1: *IN VITRO* TECHNICAL VALIDATION

Reagents

All chemicals were standard high-quality reagents from either Sigma or Merck (Darmstadt, Germany), unless otherwise stated. The phosphate buffer solution (PBS) used in the different experiments had a composition as follows: (1) coating buffer and standard buffer: 7.35 mmol/L KH_2PO_4, 40 mmol/L $Na_2HPO_4 \times 2H_2O$, 2.7 mmol/L KCl, 137 mmol/L NaCl, 10 g/L bovine serum albumin (BSA), 0.18 g/L Bronidox (Henkel, Germany), 25 mmol/L ethylenedinitrilotetraacetic acid (EDTA), and 1.1 g/L Tween 20, 0.03 g/L phenol red, pH 7.0; (2) incubation and peroxidase (POD) buffer: coating buffer plus 5% Liquid II (blocking buffer, cat no. 119941736-001, Roche, Germany), and 0.03 g/L bromphenol blue, pH 7.0; and (3) washing buffer: 25 mmol/L Tris, 50 mmol/L NaCl, 1 g/L Tween 20, pH 7.2.

Preparation of monoclonal antibodies

The monoclonal antibody (mAb) F4601 (subclass IgG_1k) was prepared exactly as described previously in detail[16]. The mAbF4601 was chosen because of its better stability data compared with F26.

Determination of antibody's specificity

Solutions of eight different peptides (KGPDP, KGPDPL, EKGPD, REKGPD, REKGPDP, EKGPDP, EKGPDPL and DEKAGGAQ (Chimex Ltd, St. Petersburg, Russia)) in the concentration range from 10^{-10} to 10^{-4} M were used for the assessment of the specificity of the mAbF4601. The Enzyme-linked immunosorbent assay (ELISA) used for these experiments had a competitive format.

Assay for detection of CTX-II fragments in serum or synovial fluid

Biotinylated F4601 antibodies were diluted in coating buffer (100 μL, 500 μg/L) and pipetted into wells of a streptavidin-coated micro-titre plate (Roche, Germany). Subsequently, the plates were placed on a mixing apparatus and subjected to shaking at 3000 rpm while incubated for 30 ± 5 min at 18–22°C (all subsequent incubations were performed under identical conditions). Thereafter, the plates were washed with buffer five times. Twenty-five microlitres of the calibrator, control, or unknown serum sample was pipetted into the appropriate micro-titre wells followed by addition of 100 μL incubation buffer. After incubation for 60 ± 5 min and washing, 100 μL of peroxidase-conjugated mAbF4601 (diluted in POD buffer) was pipetted into each well. Following incubation for another 60 ± 5 min, the wells were emptied, after which 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (Kierkegaard & Perry, Gaithersburg, MD) was added to each well. After 15 min of incubation in darkness, the colour reaction was stopped by addition of 100 μL of 0.18 mol/L H_2SO_4. The spectrophotometric absorbance was measured at 450 nm, with 650 nm as the reference wavelength, using a micro-titre plate reader (Molecular Devices, USA).

Fetal calf serum (FCS) containing a native molecule of C-telopeptide fragment of collagen type II was chosen as standard. FCS was diluted in standard buffer (zero calibrator) and standards were prepared covering a range from 0 to 270 ng/L. The standard curve was calibrated by reading the standards on a calibration curve prepared with synthetic dipeptide, i.e., EKGPDP–EKGPDP (ε-succinyl linked K, High Performance Liquid Chromatography (HPLC) purity > 95%, Pepceuticals Ltd, UK). To increase stability, the standards were freeze-dried and stored at 4°C.

Enzymatic assays for collagen type II degradation

All reactions were investigated in 0.5 ml of the matrix metalloproteinase (MMP) buffer (50 mM Tris–HCl buffer (pH 7.5), 10 mM $CaCl_2$, 150 mM NaCl and 0.05% Brij 35). Samples of 2–3 mg articular cartilage taken from the stifle joint of 9-month-old cows were submerged in liquid nitrogen and defrosted (three repeated cycles). Subsequently, the tissue samples were washed three times in MMP buffer. The MMP-9 and MMP-13 (R&D Systems, USA) enzymes were activated by co-incubation with 1 mM APMA (*p*-aminophenylmercuric acetate) for 24 h at 37°C. After activation, 10 μL of the enzyme suspension was added to a 0.490 ml aliquot of the cartilage suspension giving a final MMP concentration of 20 nM. Incubation was performed in triplicates for 24 h at 37°C under gentle shaking. At the end of the incubation, the reaction was stopped by addition of 10 μL of 10 mM GM6001 (Ilomastat, AMS Scientific, USA). The supernatants were collected and kept at −80°C until assaying by the novel immunoassay. To investigate the role of MMPs in the generation of CTX-II fragments, the tissue samples were incubated under the following conditions: (1) incubation in MMP buffer only (negative control), (2) incubation with MMPs, (3) incubation in the simultaneous presence of MMPs and the MMP inhibitor (GM6001), or (4) incubation with the MMP inhibitor only.

Lowest detection limit, intra- and inter-assay coefficient of variations (CVs), and linearity

These assay parameters were determined as described earlier by Christgau *et al.*[16]. In brief, the detection limit was defined as the concentration corresponding to 3 SD above the mean of 21 determinations of the zero calibrator. Intra- and inter-assay CVs were assessed by the measurement of 10 serum samples in 10 consecutive analytical runs, each sample measured in duplicates. Measurements

of diluted serum samples assessed the linearity of the CTX-II immunoassay. Dilution was performed in increments of 20%. Dilution recovery was expressed as percentage of the theoretically expected values. Analytical recovery was performed by spiking standards into serum samples and spiking a serum sample into another serum sample.

PART 2: *IN VIVO* BIOLOGICAL VALIDATION

Animals

Forty-six 8-week-old female Lewis (LEW/SsN/CrCrlBR) and 59 8-week-old male Sprague–Dawley CD® (Crl:CD® (SD)BR) rats were obtained from Charles River Canada Inc. (Quebec, Canada). The animals were housed (2–3 per cage) in a room maintained at 22 ± 3°C, subjected to 12-h/12-h light/dark cycles and given food (RPR 5L35, PMI Nutrition International Inc., USA) and water *ad libitum*. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and were performed according to the guidelines of the USA National Research Council and the Canadian Council on Animal Care (CCAC) and in accordance with the United States Food and Drug Administration, Good Laboratory Practice Regulations (21 CFR Part 58).

Study design

Allocation of the animals to the different intervention groups of the CIA and MIA cohorts is illustrated in Table I. For the CIA model, two cohorts were initiated to assess the joint lesions at two distinct time points, i.e., Day 22 and Day 28. For induction of CIA, bovine type II collagen (Chondrex Inc., Redmond, WA, USA) emulsified in an equal volume of incomplete Freund's adjuvant (Sigma Chemicals Co., St. Louis, MO, USA) was used. All rats in Groups 2 and 3 were immunized twice – on Day 1 (200 μL) and Day 8 (100 μL) – by subcutaneous injection to the base of the tail. The control group (Group 1) received saline injection. In Groups 5 and 6 of the MIA cohort, arthritis was induced by injecting 0.25 mg mono-iodoacetate (Sigma Chemicals Co., St. Louis, MO, USA) dissolved in 0.025 ml saline to the right femoro-tibial joint. The respective control animals (Group 4) received injection of saline in identical volume.

Dexamethasone treatment of Group 3 was initiated at the onset of the disease (defined as mild, but definite redness and/or swelling of at least one joint), whereas doxycycline treatment of Group 6 was initiated on the day of the disease induction and was continued twice daily for 7 days. Both treatments were administered as oral gavage.

On the day of termination (Day 22 or Day 28), the animals' blood was collected from the abdominal aorta using a syringe. Simultaneously, synovial fluid was collected from both femoro-tibial joints using a fine needle syringe filled with 200 μL saline. Prior assaying, synovial fluid samples were incubated overnight at 37°C in the presence of 5 units/ml hyaluronidase[24].

Macroscopic and microscopic evaluations of joint lesions

The macroscopic severity score was focused on inflammatory signs, such as redness and swelling, which were graded on a semi-quantitative scale from 0 to 4 (0: normal; 1: mild, but definite redness and/or swelling of the ankle or wrist, or apparent redness and/or swelling limited to individual digits, 2: moderate redness and/or swelling of ankle or wrist; 3: severe redness and/or swelling of the entire hind paw including digits; 4: maximally inflamed limb with involvement of multiple joints). This evaluation was performed in rats with CIA only. Given the systemic nature of the disease, the scoring included the radio-carpal, femoro-tibial, tibio-tarsal and tarsal joints of both sides.

Microscopic evaluation in rats with CIA was performed at femoro-tibial and tarsal joints. In rats with MIA, the scoring was focused on the injected femoro-tibial joint. The respective joints were collected and retained in 10% formalin. The joints were decalcified in formic acid, dehydrated through

Table I
Study design

CIA model

Group no. identification	Induction of arthritis	Intervention given for 6 days	Number of females	
			Day 22 stopped	Day 28 stopped
(1) Negative control				
A	Saline	Saline	10	
B	Saline	Saline		10
(2) Positive control				
A	Bovine CII	Saline	9	
B	Bovine CII	Saline		9
(3) Treated group	Bovine CII	Dexamethasone 0.025 mg/kg/day	8	

MIA model

Group no. identification	Induction of arthritis	Control article	Number of males
			Day 22 stopped
(4) Negative control	Saline	Deionised water	19*
(5) Positive control	Mono-iodoacetate	Deionised water	20*
(6) Treated group	Mono-iodoacetate	Doxycycline 10 mg/kg/day	20*

CII denotes collagen type II.

*From Groups 4, 5 and 6 only 13, 14 and 15 animals, respectively, underwent microscopic scoring.

graded alcohol, cleared with xylene, and embedded in paraffin. Two sections were cut: one was stained with Safranin-O and the other with haematoxylin and eosin. The severity of the joint damage was assessed microscopically for the presence of cartilage damage (fibrillations/erosions/clefts, chondrocyte necrosis, proteoglycan loss), subchondral bone resorption, osteophytes, and inflammation. Severity of each lesion was graded semi-quantitatively on a scale from 0 to 5 (0: no change, 1: minimal, 2: slight, 3: moderate, 4: severe, 5: massive). Subcores of the individual lesions were summarized in a total score. The same investigator, who was blinded for treatment codes, performed the scoring of the histological sections. A more detailed description of the used method can be found elsewhere[25–27].

STATISTICAL ANALYSIS

Differences between means of the different study parameters were compared with Kruskal–Wallis test followed by pair wise comparison of selected groups with Mann–Whitney test. Correlations of CTX-II in the serum and synovial fluid with microscopic severity scores of joint lesions were assessed using the Spearman's rank correlation analysis. Differences and associations were considered as statistically significant, if $P < 0.05$.

Results

PART 1: *IN VITRO* TECHNICAL VALIDATION

Antibody's specificity

A 50% inhibition of the ELISA signal was obtained in the presence of 90 nmol/L CTX-II peptide (EKGPDP). Extending the peptide with one N-terminal amino acid (REKGPDP) produced a similar inhibition curve. The peptide KGPDP, truncated at the N-terminal end, yielded a 50% inhibition of the ELISA signal at a concentration of 5 μmol/L. Neither the peptides EKGPDPL and KGPDPL (both being extended with one Leucin at the C-terminal), nor the peptides EKGPD and REKGPD (both lacking a free C-terminal Prolin) could inhibit the signal when investigated in concentrations up to 100 μmol/L. The mAbF4601 antibody was not reacting with the peptide DEKAGGAQ derived from the N-terminal telopeptide of type II collagen (Fig. 1).

CTX-II is generated by MMP-9 and MMP-13

Addition of MMP-9 and MMP-13 to articular explants resulted in rapid induction of collagen type II degradation and release of epitopes, which were detectable by the CTX-II assay. The release of these epitopes and hence the signal detected by the assay could be blocked by addition of an MMP inhibitor, GM6001 (Table II).

Assay performance

The calibration curve showed good linearity in the range from 0 to 270 ng/L. The lowest detection limit was 1.1 ng/L. Both intra- and inter-assay CVs were below 8.0%. The dilution recovery was in the range 83–106% with an overall mean of 99% (Table III). Similar good recoveries were found when spiking standards into serum samples or when spiking a serum sample into another serum sample (Table IV).

PART 2: *IN VIVO* BIOLOGICAL VALIDATION

Collagen-induced arthritis

Macroscopic and microscopic evaluations of joint lesions. Animals in Groups 1A and 1B showed no signs of inflammation in any of the assessed joints. In contrast, immunization of rats belonging to Groups 2A and 2B provoked pronounced local inflammation causing redness and swelling of the soft tissue around the joints. These inflammatory signs were present in the tibio-tarsal and tarsal joints of all animals (9/9), and seven of the nine animals showed it in the femoro-tibial joint. Inflammation at the radio-carpal joint was present in one animal. Treatment with dexamethasone

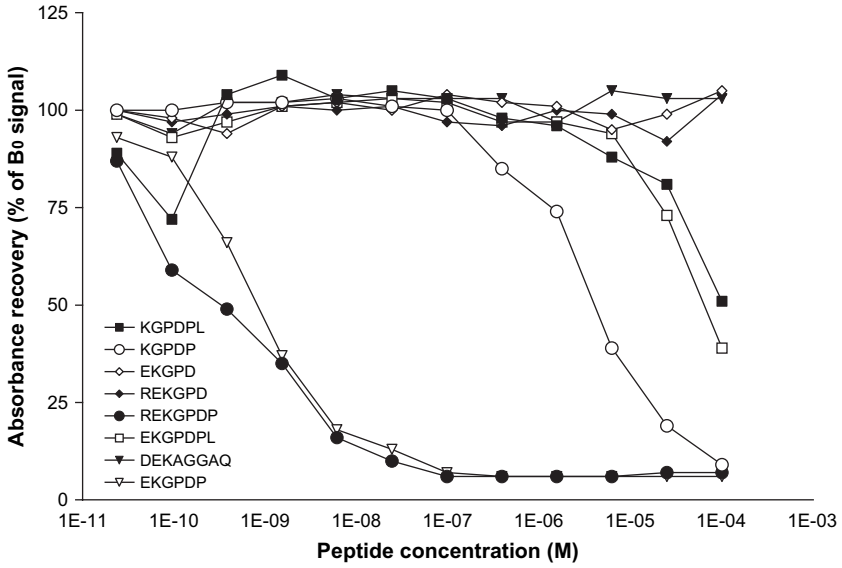

Fig. 1. Binding specificity of the mAbF4601. Specificity was evaluated by measuring synthetic peptides mimicking different parts of the C-terminal telopeptide in the α1-chain of human type II collagen, and one peptide specific for the N-terminal telopeptide (DEKAGGAQ). For all measurements, a competitive ELISA was used.

Table II
The release of CTX-II from cartilage explants under various experimental conditions evaluated by the CTX-II assay

CTX-II release (pg/ml)					
− enzyme		+ MMP-9		+ MMP-13	
− GM6001	+ GM6001	− GM6001	+ GM6001	− GM6001	+ GM6001
4.9 ± 3.2	1.6 ± 0.5	1334.9 ± 211.1	1.4 ± 1.0	4475.7 ± 852.2	5.4 ± 5.4

Addition of MMP-9 and MMP-13 resulted in the release of degradation products detectable by the assay. The enzymatic degradation could be blunted by simultaneous addition of an MMP inhibitor, GM6001. Results shown are mean value ± S.E.M.

(Group 3) did not lower the macroscopic severity scores compared to the positive control group (Group 2). Thus, the mean macroscopic severity scores at the day of disease onset for the three intervention groups were as follows: 0 for negative control group (Group 1A); 2.4 for positive control group (Group 2A) and 2.9 for the dexamethasone-treated group (Group 3).

The means of total microscopic scores for the three intervention groups followed for 22 days are indicated in Fig. 2. As indicated by the figure, the mean score of rats in Group 2A was significantly higher than that of rats in Group 1A ($P < 0.05$). The presence and severity of microscopic findings are shown in Fig. 3. When comparing the microscopic severity scores of untreated CIA rats sacrificed at Day 28 with those sacrificed at Day 22, we observed higher subscores of bone lesions, but not of cartilage lesions. Thus, mean subscores of bone lesions at Day 22 and Day 28 were 2.9 and 5.7, respectively ($P < 0.001$), whereas subscores referring to cartilage damage were 4.6 and 6.3, respectively ($P > 0.05$). Total microscopic severity scores of dexamethasone-treated animals were lower than the scores of untreated animals, but differences did not reach statistical significance.

CTX-II levels in the synovial fluid and the serum. The mean values of CTX-II in the synovial fluid for the three groups followed for 22 days are shown in Fig. 2. CTX-II levels in the immunized rats (Group 2A) were markedly higher compared to CTX-II levels in negative controls, i.e., Group 1A ($P < 0.0001$). Although CTX-II concentration in the synovial fluid from dexamethasone-treated animals was generally lower, differences compared with levels in untreated immunized rats did not reach statistical significance. CTX-II concentrations in the synovial fluid of untreated immunized animals followed for 28 days (Group 2B) were comparable with CTX-II levels of animals followed for 22 days (Group 2A).

The corresponding values of serum CTX-II are summarized in Fig. 2. The serum levels of CTX-II were also markedly elevated in untreated immunized animals (Group 2A) compared with negative controls (Group 1A, $P < 0.05$). Serum CTX-II levels in dexamethasone-treated animals (Group 3) were on an average lower than those in untreated animals (Group 2A), but differences did not reach statistical significance. In addition, serum levels of CTX-II at Day 28 (Group 2B) were comparable with those at Day 22 (Group 2A).

Association between the total microscopic severity scores of joint lesions and CTX-II. There were strong associations

Table III
Dilution recoveries

Sample	Dilution (%)	Measured concentration (ng/L)	Expected concentration (ng/L)	Recovery (%)
Serum I	None	40.5	40.5	100
	20	34.3	32.4	106
	40	25.0	24.3	103
	60	14.5	16.2	90
	80	8.2	8.1	101
	90	3.3	4.1	82
Serum II	None	57.0	57.0	100
	20	45.1	45.6	99
	40	36.2	34.2	106
	60	26.1	22.8	115
	80	12.5	11.4	110
	90	5.6	5.7	97
Serum III	None	38.8	38.8	100
	20	32.8	31.1	105
	40	23.5	23.3	101
	60	16.2	15.5	104
	80	7.6	7.8	98

Three different serum samples were measured either undiluted or diluted in standard buffer. The undiluted serum sample was set to 100%. Average recovery is 99 ± 8.2%.

Table IV
Analytical recovery for serum samples in serum CTX-II test

Sample	Spiking (ng/L)	Measured concentration (ng/L)	Expected concentration (ng/L)	Recovery (%)
Serum IV	None	28.3	28.3	100
	+ Std. A (0)	14.6	14.2	103
	+ Std. B (7)	16.8	17.3	97
	+ Std. C (17)	19.8	22.1	90
	+ Std. D (42)	31.5	34.0	93
	+ Std. E (105)	58.2	63.7	91
	+ Std. F (263)	119.3	138.0	86
Serum V	None	66.9	66.9	100
	+ Std. A (0)	34.7	33.4	104
	+ Std. B (7)	38.3	36.6	105
	+ Std. C (17)	40.6	41.3	98
	+ Std. D (42)	49.1	53.2	92
	+ Std. E (105)	76.4	83.0	92
	+ Std. F (263)	135.4	157.2	86
Serum IV + Serum V	20 + 80%	35.9	30.7	86
	40 + 60%	43.4	45.1	104
	60 + 40%	50.9	54.0	106
	80 + 20%	58.4	57.8	99

Serum samples were diluted 1:1 with calibrators and diluted in increments of 20% in another serum sample. Average recovery is 98.0 ± 7.0%. The corresponding average recovery for urinary assay is 100 ± 11.7%.

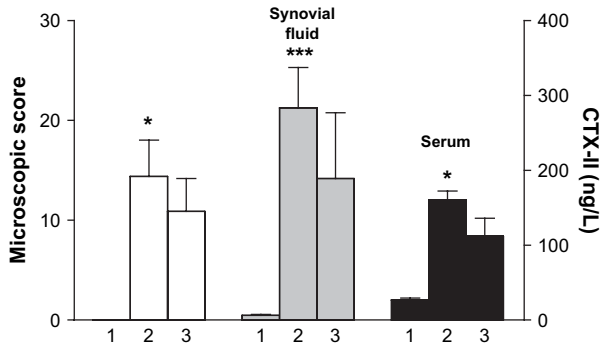

Fig. 2. Microscopic severity scores of joint lesions (white bars) and CTX-II levels in synovial fluid (grey bars) and serum (black bars) samples for negative controls (Group 1), animals with CIA (Group 2) and animals with CIA treated with dexamethasone 0.025 mg/kg/day (Group 3) at Day 22. Results are shown as mean + S.E.M. $^{*}P < 0.05$; $^{***}P < 0.001$ (compared with negative controls).

between the severity of joint damage (i.e., total microscopic severity scores) and CTX-II levels in the synovial fluid ($r = 0.76$; $P < 0.0001$) and the serum ($r = 0.85$; $P < 0.0001$). Accordingly, there was a significant association between CTX-II concentrations of synovial fluid and serum ($r = 0.83$; $P < 0.0001$). These associations are also illustrated in Fig. 4(A–C).

Mono-iodoacetate-induced OA

Microscopic findings. Microscopic alterations in the three intervention groups are indicated in Fig. 5. Intra-articular injection of mono-iodoacetate resulted in minimal to severe chondrocyte necrosis, proteoglycan loss, and subchondral bone lesions in all rats. The mean of the total severity score of these lesions in Group 5 was significantly higher compared with that in animals of Group 4 ($P < 0.001$). Total severity scores of doxycycline-treated rats (Group 6) were similar to those not receiving treatment (Group 5).

CTX-II levels in synovial fluid and serum. As expected, the monoarthritic lesion was insufficient to increase serum levels of CTX-II. However, measurements in the synovial fluid indicated elevated levels of CTX-II in the femoro-tibial joints injected with mono-iodoacetate (Group 5) compared with those injected with saline only (Group 4, $P < 0.05$). CTX-II levels in the synovial fluid from doxycycline-treated

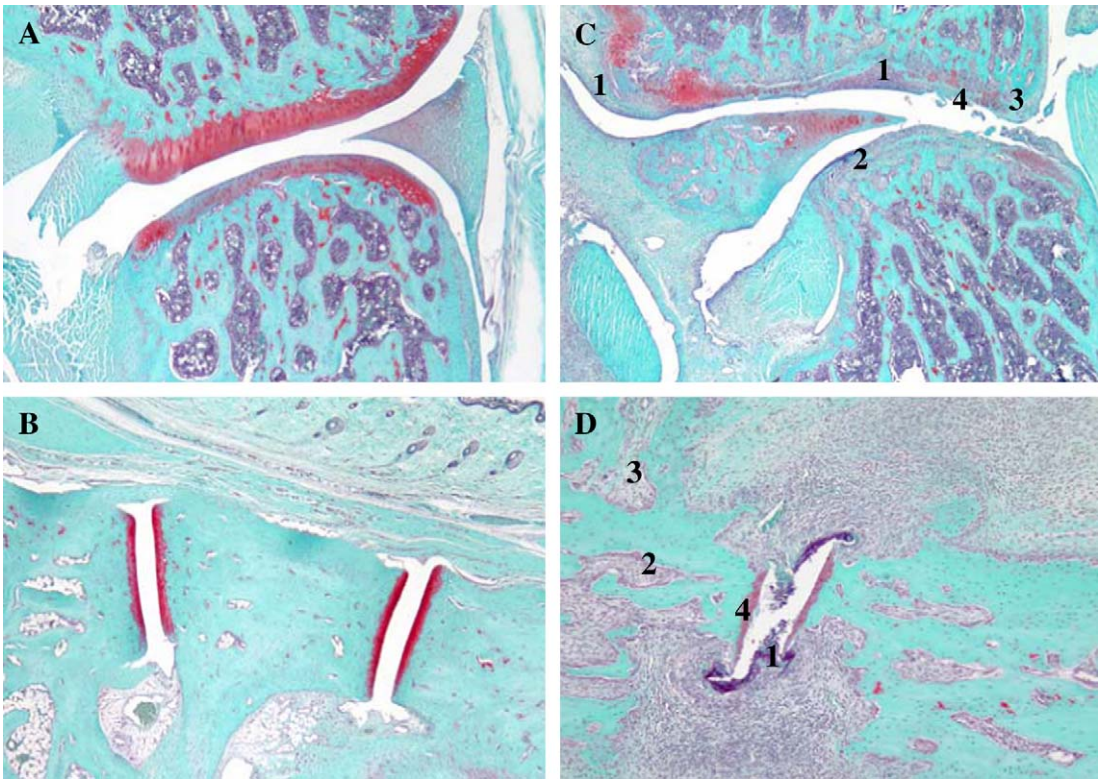

Fig. 3. The microscopic findings in the femoro-tibial (A, C) and tarsal (B, D) joints from an animal with CIA (right panel) with reference to a negative control animal (left panel). Sections were stained with Safranin-O for visualizations of proteoglycans (2× magnification). Lesions illustrated are (1) inflammation; (2) bone resorption; (3) osteophyte; and (4) cartilage damage. Each of these was graded on a scale from 0 to 5. The femoro-tibial joint illustrated in Panel C received the following subscores: 3 for inflammation; 4 for bone resorption; 1 for osteophyte and 3 for cartilage damage. The tarsal joint illustrated in Panel D from the same animal received a subscore of 4 for all parameters. Thus, the total score was 27. The animals from the negative control group illustrated in Panels A and B received a total score of 0. The serum CTX-II values for the negative control and the untreated CIA rat were 26.9 pg/ml and 163.2 pg/ml, respectively. The corresponding levels of CTX-II in the synovial fluid were 7.2 pg/ml and 274.8 pg/ml, respectively.

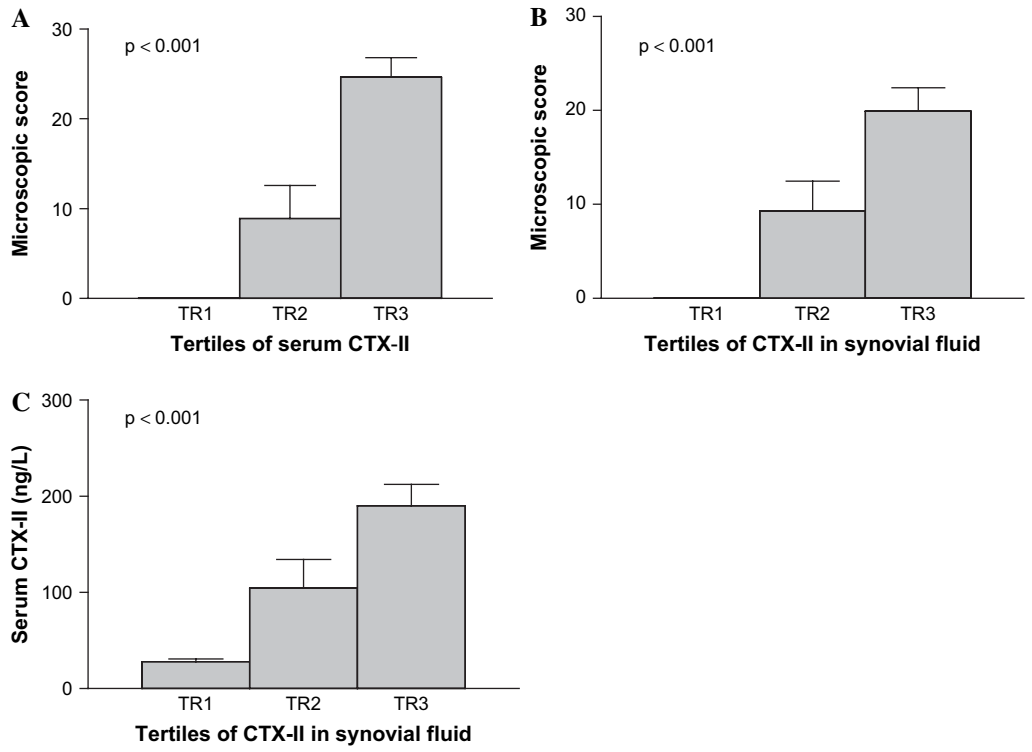

Fig. 4. Association between CTX-II level and the total microscopic severity score of joint lesions in the CIA model. Means of total microscopic severity scores in tertiles of serum CTX-II at Day 22 (Panel A), means of total microscopic severity scores in tertiles of CTX-II in the synovial fluid at Day 22 (Panel B), and means of serum CTX-II in tertiles of CTX-II in the synovial fluid at Day 22 (Panel C). Results are shown as mean + S.E.M. Cut-off values between the tertiles of serum CTX-II were 36 ng/L (33rd percentile) and 160 ng/L (66th percentile). The respective values for CTX-II in the synovial fluid were 8 ng/L (33rd percentile) and 249.6 ng/L (66th percentile).

animals were not significantly different from those in untreated animals.

Association between the total microscopic severity scores of joint lesions and CTX-II. CTX-II in the synovial fluid showed statistically significant correlations with the total microscopic severity scores of joint lesions ($r = 0.53$; $P < 0.0001$). The linear association between these parameters is also illustrated in Fig. 6.

Discussion

In the present study, we have shown that F4601 is specific for the EKGPDP sequence, which is an epitope exclusively present in collagen type II. The F4601 antibody similar to the F26 antibody[16], which is used in the urinary CTX-II (CartiLaps) assay binds to the free C-terminal Proline. However, whereas the urinary CTX-II assay is a competitive ELISA that recognizes monomer and cross-linked

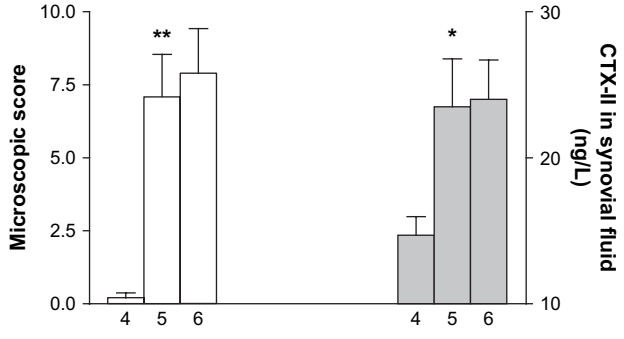

Fig. 5. Means of the microscopic severity score of joint lesions (white bars) and CTX-II in the synovial fluid (grey bars) in control animals (Group 4), animals with MIA (Group 5) and animals with MIA treated with doxycycline 10 mg/kg/day (Group 6) at Day 22. Results are shown as mean + S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$.

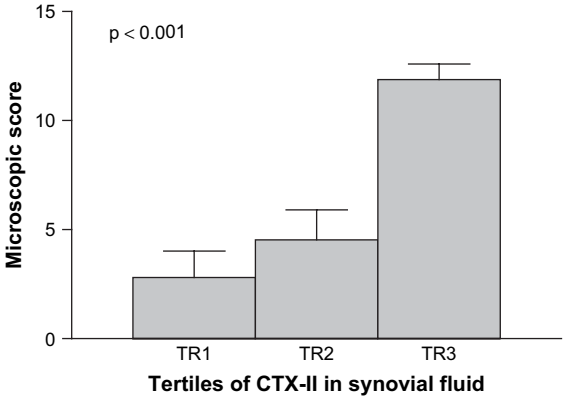

Fig. 6. Association between CTX-II level in the synovial fluid and the microscopic severity scores of joint lesions in the MIA model. Results are shown as mean + S.E.M. Cut-off values between the tertiles of CTX-II were 18 ng/L (33rd percentile) and 28.4 ng/L (66th percentile).

dimer CTX-II, the herein described assay is a sandwich assay that targets dimer CTX-II only.

An important question is whether the CTX-II epitope is generated during enzymatic degradation of hyaline cartilage *in vivo*. Chondrocytes and monocytes/macrophages can mutually induce alterations in the expression of catabolic enzymes, in particular, enzymes of the MMP family. MMP-9 and MMP-13 have recently been implicated in cartilage degradation of RA patients[28,29]. When articular explants were subjected to enzymatic degradation by MMP-9 or MMP-13, we found strong signals detected by the assay indicating the generation of epitopes. When inhibiting MMPs, the generation of epitopes was no longer present. Collectively, these findings suggest that the Preclinical Serum CTX-II assay detects peptide fragments, which are generated during enzymatic processes with direct *in vivo* relevance.

The Preclinical Serum CTX-II assay demonstrated a high degree of precision and reproducibility with inter- and intra-assay CVs below 8.0%, with good linearity. Furthermore, when the calibrator was spiked into a serum sample or when one serum sample was spiked into another serum sample, the antigen was measured with recovery rates close to 100%, indicating that the antibody has similar affinity for the calibrator and the authentic antigen in the serum. Regarding these technical details, the performance of the serum assay is comparable with those provided by the previously introduced urinary CTX-II assay[16].

Our *in vivo* experiments investigated whether measuring CTX-II in serum and synovial fluid samples can provide information about the structural integrity of articular cartilage in two models of arthritis. The main findings were as follows: (1) CTX-II levels in the synovial fluid showed significant correlation with the total microscopic severity scores of joint lesions in both models, (2) serum CTX-II showed strong association with the respective total microscopic severity scores in the CIA model as well as with CTX-II levels in the synovial fluid; no similar associations were revealed in the MIA model, and (3) differences in CTX-II in the synovial fluid between untreated and treated animals followed the same pattern as shown by differences in the total microscopic severity scores of joint damage at the end of the treatment period in both arthritis models (this was also true for serum CTX-II levels in the CIA model). Collectively, these findings suggest that the Preclinical Serum CTX-II ELISA could provide a reliable non-invasive assessment of disease progression and therapeutic responses to candidate drugs with chondroprotective potentials.

CIA in rodents shares histological and immunological features of human RA and therefore is currently one of the most preferred experimental models[1,3,4,30]. Several markers, such as COMP, C2C, and urinary CTX-II have been tested for their utility to monitor cartilage damage in the CIA model. However, as reported by Larsson *et al.*[11] "there was only a relatively limited increase in the serum levels of COMP at day 21 when the rats had already developed high arthritis scores, whereas the main increase in serum levels of COMP appeared between days 21 and 28". Measurements of C2C levels in the CIA model also failed to show significant differences between positive and negative control groups[31]. In the present study, the Preclinical Serum assay detected marked and statistically significant increases in CTX-II both in serum and in synovial fluid samples collected at Day 22. The CTX-II measurements could not only discriminate control and diseased groups, but could also give quantitative information regarding the severity of joint damage. This latter feature was indicated by a significant correlation between total microscopic severity scores of joint lesions and the level of CTX-II in the serum or the synovial fluid. Thus, these results suggest that the herein introduced assay allowing measurements of CTX-II in synovial fluid and/or serum samples provides a useful diagnostic tool for the non-invasive assessment of cartilage status in the CIA model.

The CIA model is often used to evaluate the chondroprotective effects of disease-modifying agents. For example, corticosteroids were repeatedly shown to provide therapeutical benefits in inflammatory joint diseases[12,30,32]. Our findings indicated lower microscopic scores and lower levels of CTX-II in the synovial fluid and serum of animals receiving dexamethasone treatment. However, when compared to previously published data[20], the inhibition achieved was notably weaker, likely due to insufficient dosing. This was also indicated by the lack of anti-inflammatory effects as assessed by the impact on redness and swelling of the joints. Nevertheless, CTX-II levels adequately reflected the modest attenuation of structural damage by the treatment.

Doxycycline, similar to more specific inhibitors of MMPs, was found to provide therapeutical benefits in the MIA model[33]. The treatment applied in the present study failed to elicit effective prevention of accelerated cartilage turnover and the consequent structural alterations. The lack of efficacy could be due to an imbalance between the intensity of disease and the selected dose or overall duration of the treatment. Notwithstanding this failure, the lack of efficacy was again adequately reflected by the CTX-II measurement in the synovial sample, further emphasizing the utility for estimating structural status of articular cartilage.

Circulating levels of CTX-II showed poor to no association with the severity of joint lesions in the MIA model. This can be explained by differences in the pathogenesis of the joint disease. In the CIA model, animals are immunized with bovine collagen type II, which generates a systemic inflammatory response and utilization of an array of enzymatic processes to eliminate collagen type II. In contrast, in the MIA model, only a monoarthritic lesion is provoked by local metabolic paralysis of chondrocytes. The difference in the intensity of the two forms of arthritis is also indicated by the observation that CTX-II levels in the synovial fluid were ~10-fold higher in rats with CIA compared with levels in rats with MIA. These differences in intensity and extent (polyarthritis vs monoarthritis) of the two joint diseases seem to provide a plausible explanation to why serum levels are more informative in the CIA model.

The results of the study suggest that the herein introduced assay provides a useful diagnostic tool for measuring CTX-II in both serum and synovial fluid samples from rodents, which in turn might facilitate the non-invasive assessment of articular cartilage status in these animals. Importantly, whereas CTX-II in the synovial fluid adequately reflected cartilage status of the affected joint in both models, the utility of serum measurements seems to be limited to systemic forms of joint diseases. These findings call for intervention studies investigating whether early, treatment-induced changes in serum CTX-II can predict the chondroprotective potentials of candidate drugs.

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