Serological CTX-II does not measure the same as urinary CTX-II

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SUMMARY

Objective: Type II collagen is the most abundant protein of articular cartilage. The urinary cross-linked C-terminal telopeptide of type II collagen (uCTX-II) is a matrix metalloproteinase (MMP) cleaved fragment and may be the most well-validated biomarker in osteoarthritis. The aim was to develop a serological immunoassay of CTX-II (sCTX-II) and evaluated both sCTX-II and uCTX-II levels in a cross-sectional osteoarthritis cohort.

Methods: The biological relevance of sCTX-II was validated in bovine cartilage explants cultured in the presence of Oncostatin M and tumor necrosis factor alpha (OT) or OT supplemented with GM6001 for 3 weeks. Serum and urine samples from an osteoarthritis cohort were assayed using sCTX-II and uCTX-II, respectively. Spearman’s correlation was performed to evaluate the correlation between sCTX-II and uCTX-II. The association between the level of biomarkers and clinical variables was also investigated.

Results: The supernatant analyzed in sCTX-II showed significantly higher CTX-II levels in the end phases of explant culture compared to the vehicle group. The release of CTX-II was completely suppressed by GM6001. The sCTX-II levels in serum were not associated with uCTX-II in urine although sCTX-II levels in urine were significantly correlated with uCTX-II. uCTX-II correlated with age and gender while sCTX-II did not. sCTX-II cannot demonstrate any clinical relevance in a cross-sectional OA cohort as uCTX-II did.

Conclusion: The sCTX-II assay can reflect the MMP-mediated type II collagen degradation in bovine cartilage explants. However, sCTX-II and uCTX-II assays show different patterns suggesting the presence of CTX-II in blood may be different from that of urine.

1. Introduction

A hallmark of osteoarthritis (OA) is the degradation of the extracellular matrix (ECM) in articular cartilage. Type II collagen is the most abundant protein of articular cartilage and is highly specific for this tissue. Numerous studies have proven that cartilage, especially type II collagen, degradation is an essential step in the progression of knee OA [1,2].

The CTX-II is a cross-linked C-terminal telopeptide of type II collagen and contains a dimeric-hexapeptide epitope (EKGPDP) with a pyridine ring as a linker, which is cleaved by matrix metalloproteinases (MMPs) [3-5]. CTX-II diffuses from the joint to blood and is ultimately excreted into the urine. Urinary CTX-II (uCTX-II) was originally described by D. Eyre [3] and later on, a commercial assay was launched in 2001 [6]. It has been suggested to be the most tested and best validated biochemical marker for assessing collagenolysis in articular cartilage during OA [7–10]. uCTX-II is elevated in knee OA [11,12], hip OA [13,14] and rheumatoid arthritis (RA) [15,16] patients when compared to healthy subjects. Patients with higher baseline CTX-II levels have a greater risk of knee OA progression [12,17]. Additionally, uCTX-II is associated with knee pain [18] although pain associations have varied [19]. Furthermore, uCTX-II can predict the effectiveness of anti-inflammatory therapy in knee OA [20], even though no predictions are seen in other trials [21]. OA patients with high uCTX-II levels showed markedly decreased uCTX-II after treatment with nimesulide but not ibuprofen [20]. Although the lack of approved disease-modifying osteoarthritis drugs (DMOADs) limited the clinical use of uCTX-II, uCTX-II may be applied in early phase evaluation of the efficacy of DMOADs, OA progression and monitoring health status in the general population [22].

It has been reported that both serological CTX-II (sCTX-II) and uCTX-II levels are related to arthritis onset and cartilage destruction in rats with collagen-induced arthritis, suggesting sCTX-II are in line with uCTX-II in rats [23,24]. However, knowledge about sCTX-II levels in human is limited as no comprehensive study has been reported to date. Whether this is due to the lack of high sensitivity in the current uCTX-II assay is unclear. Considering serum may have less analytic and biologic...
variability than urine, it is warranted for the development of sCTX-II immunoassay in human. Thus, in this study, we aimed to develop a sCTX-II assay by using the same antibody from uCTX-II (Urine CartiLaps ELISA, IDS, UK) on a highly sensitive electrochemiluminescent platform to determine the levels of CTX-II in blood and to investigate sCTX-II as well as uCTX-II in a cross-sectional OA cohort.

2. Methods

2.1. Materials

All chemicals were bought from either Sigma-Aldrich or Merck unless otherwise stated. 96-well Gold streptavidin microtitre plate, Sulfo-TAG labeling kit, 4x read buffer T with a surfactant, and QuickPlex SQ 120 reader with Discovery Workbench software was purchased from Meso Scale Diagnostics (Gaithersburg, USA).

2.2. Assay protocol of sCTX-II ECLIA

A sandwich assay was developed using the electrochemiluminescence (ECL) technology on the Mesoscale Discovery (MSD) platform [25]. The antibody was the same as the one employed in uCTX-II ELISA. Briefly, the MSD Gold 96-well streptavidin plate was blocked with 100 μL of blocking buffer (10 mM PBS, 5% BSA, pH 7.4) and incubated for 1 h at 20 °C. After 3 times washing, the plate was coated with 3 μg/mL of the biotinylated antibody, dissolved in assay buffer (50 mM PBS, 1% BSA, 0.1% Tween-20, 8 g/L NaCl, 0.93% Tritiplex® III, 5% Liquid II, pH 7.4) and incubated for 1 h at 20 °C. The plate was washed three times and 50 μL of the fetal bovine serum calibrators or samples were added to the appropriate wells. The plate was incubated overnight at 2–8 °C. Then 25 μL of 4 μg/mL Sulfo-TAG-labeled detection antibody was added into the plate followed by incubation for 1 h at 20 °C. Finally, 150 μL 2x Read buffer T was added, and the plate was read immediately on a MESCO QuickPlex SQ 120 reader. All the above incubation steps included shaking at 300 rpm. After each incubation step, the plate was washed three times with 10 mM PBS ±0.05% Tween-20, pH 7.4.

2.3. Technical evaluation of sCTX-II ECLIA

The intra-assay and inter-assay coefficient of variation (CV) was calculated as the mean value of the variation of five samples analyzed 10 times in duplicate. The dilution recovery was assessed in three individual human serum samples which were diluted by the assay buffer in increasing concentrations of 10% and measured in the sCTX-II ECLIA. The measuring range was defined as the range between LLOQ (lower limit of quantification) and ULOD (upper limit of detection). The concentrations determined for the diluted samples corresponded well with the back-calculated values. Similarly, it was assessed as to whether the serum could be spiked into the serum. Three human serum samples were diluted in increments of 20% in assay buffer before spiked with another human serum in the ratio of 1:1, and the measured concentrations expressed as a percentage of the expected values.

2.4. Biological validation of sCTX-II ECLIA in bovine cartilage explants model

The bovine articular cartilage explants model were set up as previously described [26]. Briefly, full depth cartilage explants were isolated from the medial femoral condyle of cattle aged 1–2 years bought from the local slaughter (Haraldf Hansens Slagter, Slangerup, Denmark). The cartilage explants were incubated in DMEM/F-12 (Life Technologies, US) medium with 1% penicillin and streptavidin in 96-well plate at 37 °C, 5% CO2. There were six replicates for each treatment: 1) medium alone (WO); 2) catabolic stimulation with 10 ng/mL Oncostatin M and 20 ng/mL tumor necrosis factor alpha (TNF-α) (OT); 3) OT supplemented with 10 μM GM6001 (OT+GM6001). The model was cultured for 21 days. The conditioned medium was changed every two or three days, and the supernatant was stored at –20 °C until use.

2.5. Clinical evaluation of sCTX-II and uCTX-II assays in OA cohort

As reported previously [27, 28], the C4Pain cohort consisted of 281 patients with different intensity of knee joint pain. The maximal pain during the last 24 h was rated on a 10-cm continuous 0 to 100 visual analog scale (VAS) (0: no pain, 100: maximum pain). Since some samples were run out, only 245 patients were included in the present study (Fig. 1). Among the 245 participants, 188 had a diagnosis of primary knee OA according to the American College of Rheumatology (ACR) criteria [29], while 57 subjects with Kellgren-Lawrence (KL) grades ≤ 1 was defined as Non-OA control. Blood and urine were collected upon overnight fasting prior to surgery or during the consultation. All participants provided informed consent prior to enrollment, according to the Declaration of Helsinki. The study was approved by The Ethical Committee of Northern Jutland (VEK no.: N-20100094).

Biomarkers of sCTX-II and uCTX-II were analyzed in both serum and urine. uCTX-II was measured using the Urine CartiLaps (IDS, UK), which is a competitive enzyme-linked immunosorbent assay (ELISA) [6]. The analyses were performed according to the manufacturer’s instructions. Briefly, biotinylated, synthetic CTX-II peptides are bound to the surface of streptavidin-coated wells of the microtitre plate. After washing, standards, controls, and urine samples are pipetted into the wells followed by addition of a solution of the monoclonal antibody. The wells are washed, and a solution of peroxidase-conjugated rabbit anti-mouse immunoglobulin is added to the wells. Following the second washing, tetramethylbenzidine (TMB) is added to all wells and the colour reaction is stopped with 0.18 M sulfuric acid and the absorbance is measured. The concentration of uCTX-II and sCTX-II in urine was normalized to the urine creatinine by using the QuantiChrom Creatinine Assay Kit (BioAssay Systems, US). Samples falling above the ULOD were re-assayed at greater dilutions. Undiluted samples falling below LLOQ were imputed by being assigned half the LLOQ as its measured concentration.

2.6. Statistical analysis

Results are presented as the mean ± SEM. GraphPad Prism software (version 7.01) was used for all the statistical analysis except for the adjustment of body mass index (BMI), sex and age by MedCal (version 15). Differences between mean values were compared by the non-parametric Kruskal-Wallis test. The association between the level of Biomarkers of sCTX-II and uCTX-II, demographic variables were analyzed by non-parametric Spearman’s correlation. Patient tertiles of equal size depending on the sCTX-II and uCTX-II levels were made, and the difference in clinical parameters was investigated.

3. Results

3.1. Technical performance of sCTX-II ECLIA

The characterization of the antibody used in sCTX-II ECLIA was described before [6, 23]. The technical performance of this ECLIA and the sCTX-II ELISA was summarized in Supplementary Table 1. The intra-assay CV was 3%, and the inter-assay CV was 11%. The measurement range was 0.005–0.68 ng/mL. The limit of detection (LOD) defined as the concentration corresponding to 3 SD below the mean of 21 determinations of the zero calibrator was 0.003 ng/mL. The mean dilution and spiking recovery tested in human serum were 92% and 106% respectively, which were within the measurement range of the assay (100 ± 20%).
3.2. Measurement of CTX-II fragments in the bovine cartilage explants model by sCTX-II ECLIA

Due to the same immunogen sequence expected in bovine, and easy access to bovine cartilage, we investigated the specificity of the sCTX-II ECLIA by using the bovine articular explants model. The explants were cultured for 21 days ex-vivo in the presence of Oncostatin M plus TNF-α (OT), which is known to induce MMP-mediated cartilage degradation. The supernatant analyzed in the sCTX-II ECLIA showed significant higher CTX-II levels in day 17 and 19 compared to the vehicle group ($P < 0.001$, Fig. 2). In contrast, when OT co-cultured with GM6001, which is known as a general MMPs inhibitor, the release of CTX-II was completely suppressed in comparison to the OT group ($P < 0.001$, Fig. 2).

3.3. Subjects characteristics

All the subjects were divided into four groups according to their KL grades and VAS scores. There was no marked difference in sex, age, BMI, race, and serum CTX-II levels across the groups except that the age in the pain group was a bit lower when compared to the healthy controls (Table 1). The pain scores (VAS and WOMAC) were significantly higher in the pain group and the radiographic OA (ROA) with pain group ($P < 0.0001$). The KLG in ROA group either with pain (ROA + S) or without pain (ROA - S) were significantly higher than that of the healthy controls.

3.4. Associations between biomarkers and demographics

To investigate the relations between biomarkers and demographic variables, Spearman’s correlation was performed with biomarkers (sCTX-II either measured in serum or urine, uCTX-II measured in urine) as the dependent variable, and gender, age, and BMI as independent variables. Both sCTX-II levels in urine and uCTX-II levels were significantly associated with gender (Table 2), which was consistent with the previous report [30,31]. In contrast, sCTX-II levels in serum had no association with gender but negatively associated with age. In the corresponding plots (Fig. 3), mean concentrations of the sCTX-II tended to decrease with age, with minimum values seen in participants aged 70–79. On the contrary, the uCTX-II levels slightly elevated with increasing age in both genders from 40 years and upwards. Particularly, the uCTX-II concentration in women aged 70–79 was significantly higher in comparison to that in men ($P < 0.05$, Fig. 3). In the corresponding plots (Fig. 3), mean concentrations of the sCTX-II tended to decrease with age, with minimum values seen in participants aged 70–79. On the contrary, the uCTX-II levels slightly elevated with increasing age in both genders from 40 years and upwards. Particularly, the uCTX-II concentration in women aged 70–79 was significantly higher in comparison to that in men ($P < 0.05$, Fig. 3).
3.5. Relations between sCTX-II and uCTX-II

To evaluate the specificity of the sCTX-II assay, the correlation between sCTX-II and uCTX-II was analyzed by Spearman's correlation. The sCTX-II levels in serum were not associated with uCTX-II in urine and sCTX-II in urine ($r = 0.0119$, $r = -0.0225$, respectively, Table 3). Interestingly, sCTX-II levels in urine were significantly correlated with uCTX-II ($r = 0.4600$, Table 3), suggesting that to some degree the sCTX-II ECLIA detected the same analytes in urine as uCTX-II did.

3.6. Associations between sCTX-II/uCTX-II and clinical variables

uCTX-II concentrations were significantly elevated in the subjects with higher KLG compared to the ones with lower KLG (Fig. 4, B+D). By Table 3, associations between uCTX-II and sCTX-II as assessed by Spearman's correlation analysis. Spearman's correlation coefficients ($r$) and significance values ($p$) are reported.
Fig. 4. Serum CTX-II levels (A+E+G+I) and urinary CTX-II levels (B+F+H+J) depending on the KL grades and VAS pain scores. Data are shown as mean ± SEM, and the non-parametric Kruskal-Wallis test is used to test for significance. The p-values are adjusted for age, BMI, and sex. Asterisks indicate the following: *P < 0.05, **P < 0.01, and ***P < 0.001, ****P < 0.0001. BMI: body mass index; CTX-II: cross-linked carboxyl-terminal telopeptide of type II collagen; KLG: Kellgren-Lawrence grades; ROA+S: radiographic OA with pain; ROA-S: radiographic OA without pain; VAS: visual analog scale.
contrast, the sCTX-II levels decreased gradually when the KLG increases although not significantly (Fig. 4, A–C). uCTX-II was able to discriminate the ROA patients from the non-OA controls (Fig. 4, F). Meanwhile, there was a significant difference in uCTX-II between ROA+S group and healthy control (P < 0.05, Fig. 4, H). However, sCTX-II did not demonstrate such abilities (Fig. 4, E–G). In addition, we assessed the associations between pain scores and biomarkers. Unfortunately, neither sCTX-II nor uCTX-II was associated with VAS pain (Fig. 4, I–J).

We further examined the relationship between biomarker tertiles (sCTX-II and uCTX-II) and the difference in clinical parameters. The KL grades, VAS pain, WOMAC pain, stiffness and function scores were significantly higher in the highest uCTX-II tertile (T3) compared to the lowest tertile (T1) (Supplementary Fig. 1-B). uCTX-II pain, stiffness and function scores were significantly higher in the highest uCTX-II tertile (T3) compared to the lowest tertile (T1) (Supplementary Fig. 1-B). In contrast, the reverse trend was observed in sCTX-II tertiles although no significances were observed (Supplementary Fig. 1-A–C+E+G+I).

4. Discussion

We here developed an immunoassay, which is capable to analyze CTX-II concentrations in human sera. The main findings were: 1) The specificity of the sCTX-II ECLIA was evaluated in a bovine cartilage explant model. 2) sCTX-II levels in serum were not associated with uCTX-II in urine although sCTX-II levels in urine were significantly correlated with uCTX-II. 3) uCTX-II correlated with age and gender while sCTX-II did not. The age-dependent pattern in uCTX-II showed noteworthy differences with that of sCTX-II. 4) sCTX-II cannot demonstrate any clinical relevance in this OA cohort, suggesting the CTX-II protocol is not clinically applicable has nothing to do as the sand-
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