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Evaluation of cartilage damage by measuring collagen degradation products in joint extracts in a traumatic model of osteoarthritis

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Abstract

The objective of this work was to investigate whether collagen degradation products in protein extract from joints could provide quantitative information on cartilage damage. Osteoarthritis (OA) was surgically induced in rat knee joints. Joints were isolated 7, 14 and 28 days after surgery for protein extraction and histology. C-terminal telopeptide of type II collagen (CTX-II), CTX-I and hydroxyproline were measured in protein extracts. Matrix metalloproteinase (MMP)-2 and -9 activity was evaluated by gelatinase zymography and joint pathology was visualized by histology and immunohistochemistry. The results showed that levels of CTX-II were significantly increased in anterior cruciate ligament transection (ACLT)-operated compared with sham-operated knee joints on days 7 and 28, whereas the levels of hydroxyproline and CTX-I epitopes showed no difference. MMP activity was slightly increased in ACLT-operated joints. The CTX-II epitope was highly expressed and co-localized to damaged articular cartilage in ACLT-operated joints. We have therefore demonstrated an increased type II collagen degradation in knees after surgical induction of OA, and propose assessment of collagen degradation epitopes as a quantitative measure of cartilage damage.

Keywords: Osteoarthritis, anterior cruciate ligament transaction, cartilage damage, protein extraction, CTX-II, CTX-I

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Introduction

Osteoarthritis (OA) is a degenerative joint disease in which degradation of the articular cartilage results in destruction of the joint structure (Simon & Jackson 2006). Novel disease-modifying compounds are continuously evaluated, but the development and identification of new treatments is hampered by the lack of fast and reliable procedures for monitoring and evaluating disease status.

One animal model having a major role in the drug discovery process in OA is the anterior cruciate ligament transection (ACLT) model, which has been studied widely

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in various species, e.g. rabbits (Chang et al. 1997, Amiel et al. 2003), dogs (Smith et al. 2002, El Hajjaji et al. 2004), and more recently in the rat (Stoop et al. 2001, Hayami et al. 2004). In this model, the first changes include loss of superficial chondrocytes and a slight fibrillation of the articular cartilage with loss of proteoglycans, followed by cartilage erosion and collagen degradation at a later time point (Stoop et al. 2001). Denatured type II collagen has been shown only in fibrillated areas, but not in underlying cartilage (Stoop et al. 2001).

Cartilage degradation in OA is considered to be mediated mainly by two enzyme families: the matrix metalloproteinases (MMPs) which are responsible for cartilage collagen breakdown; and the family of 'a disintegrin and metalloproteinase domain with thrombospondin motifs' (ADAMTS) which mediates cartilage aggrecan breakdown. Gene expression of both enzyme families is upregulated in OA cartilage (Davidson et al. 2006), as is the enzymatic activity both in vitro and in vivo (Tang 2001, Parks et al. 2004). The action of these endopeptidases results in release of degradation neo-epitopes that originate from within the joint and are subsequently released into the circulation; among these is the MMP-derived collagen type II degradation marker C-terminal telopeptide of type II collagen (CTX-II) (Sondergaard et al. 2006a).

The traditional, often very time-consuming, methods for assessment of joint pathology in OA models include the histological evaluation of proteoglycan loss, cartilage degradation and erosion, osteophyte formation and subchondral bone changes (Pritzker et al. 2006) as well as immunohistochemistry for the demonstration of specific participants such as cytokines and catabolic enzymes. As biomarkers originating from a sole inflicted joint might be difficult to distinguish from background levels when measured in urine or serum, measurements in protein extracts from whole joints might provide an accurate and quantitative measure of tissue conditions within signal joints.

The aim of this study was to investigate the use of local concentrations of MMP-generated degradation products as a surrogate marker of bone and cartilage conditions. This method might constitute a fast and efficient method to monitor cartilage and bone degradation during drug development.

Methods

Animals and diet

Twenty-four Wistar rats (150–175 g, Taconic, Germany) were fed with a standard diet and had access to MilliQ water ad libitum. Animals were housed in Scantainer-plus (Scanbur, Sweden) cages with sawdust bedding and a 12 h light/dark cycle. Before the start of the experiment, animals were allocated into appropriate groups according to their weights.

Induction of anterior cruciate ligament transection

Rats were anaesthetized by a subcutaneous Hypnorm/Dormicum injection, and the area around the knee was shaved and disinfected with iodine. One knee was subjected to an ACLT operation, while a sham operation was performed in the contralateral knee. In the ACLT-operated knee, the joint capsule was opened by an insertion on the medial side next to the patella, thereby providing access to the joint space by dislocating the patella laterally with the leg in extension. The anterior cruciate ligament was transected using a custom-made retrograde cutting hook. After

relocation of the patella, the wound was closed with vicryl 5/0 (polyglactin 910)-braided absorbable suture (Ethicon, Edinburgh, UK). The skin was closed with staples. Care was taken to keep the operation area moist with saline during the procedure. Eighty microlitres of buprenorfinehydrochloride s.c. (Temgesic®; Reckitt & Colman Products, Kingston-upon-Hull, UK) was given as an analgesic on the day of operation and the following 3 days. This procedure is modified from the protocol described by Williams et al. (1982).

All procedures were approved by the Danish Animal Experiments Inspectorate.

Protein extraction from knee joints

The procedure for extraction of proteins was modified from Stolina et al. (2005). In detail, knee joints were isolated following euthanasia, snap frozen in liquid nitrogen and stored at -80°C until use. Still frozen, the tibia and femur were cut 3 mm from the joint, producing samples of 500–700 mg. These were re-frozen in liquid nitrogen and placed in a Bessman tissue pulverizer (Spectrum, the Netherlands). The sample was crushed and transferred to 3 ml of extraction buffer (50 mM Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl, 0.1% Triton X-100, 1 tablet/10ml buffer of Complete Mini EDTA-free protease inhibitor cocktail (Roche) and 10 μM GM6001 (Biomol). Tissues were homogenized for 2×30 s using an OMNI homogenizer, speed level 4, then the sample was centrifuged at 1700g for 10 min, supernatants were harvested and centrifuged again at 10 000g, whereafter supernatants were stored at -20°C.

Enzyme-linked immunosorbent assay

Levels of C-terminal telopeptide of type I collagen (CTX-I) and CTX-II in protein extracts from joints were measured with the RatLaps™ and CartiLaps® enzyme-linked immunosorbent assays (ELISAs), respectively (Nordic Bioscience, Herlev, Denmark), following the instructions provided by the manufacturer.

Hydroxyproline measurements

Hydroxyproline in protein extracts was measured using a procedure previously described (Sondergaard et al. 2006a). Briefly, a 10 μl sample was diluted five times in 7.5 M HCl and hydrolyzed for 20 h at 110°C, and the liquid was evaporated overnight at 50°C. The precipitate was dissolved in 250 μl 50% isopropanol in MilliQ, whereafter a 10 μl solution was mixed with 20 μl isopropanol and 10 μl reagent A (Reagent A (1:4) of 24 M chloramine T and 1 M Na acetate, 0.33 M Na_3 citrate, 0.07 M citric acid dissolved in isopropanol). The mixture was incubated for 4 ± 1 min at room temperature and added to 130 μl of reagent B (Reagent B (3:13) of 4.5 M 4-dimethylamino-benzaldehyd dissolved in 60% perchloric acid and isopropanol). The samples were incubated for 25 min at 60°C and absorbance was read at 558 nm on an ELISA reader. Standards were prepared from 1-hydroxyproline diluted in 1 mM HCl.

Gelatinase zymography of MMP activity

Protein extracts from ACLT- or sham-operated knee joints were diluted 1:5 in PBS and 10 μl + 12.5 μl sample buffer (25% 1 M Tris/base pH 6.8, 40% glycerol, 80 mg ml^{-1}

SDS, 0.5 mg ml^{-1} bromphenol blue (Merck)) was loaded on a 7.5% SDS-polyacrylamide gel containing 0.5 mg ml^{-1} gelatin (Sigma-Aldrich, UK) as a substrate. Five microlitres of rainbow marker (Amersham, USA) and gelatinase zymography standards for MMP-2 and -9 (Chemicon, USA) were loaded along on the gels, and the proteins separated. After electrophoresis, gels were washed three times with 2.5% Triton X-100 in water and incubated overnight at 37°C in incubation buffer (0.1% TritonX-100, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 3 mM NaN_3, 50 mM Tris pH 7.4) in a closed container. Gels were stained for 30 min with 0.25% Coomassie R-250 (Sigma-Aldrich) in 10% acetic acid and 45% methanol and destained for 30 min with 20% acetic acid, 20% methanol, 17% ethanol, 0.6% diethylether. Images were obtained by an Olympus C5050 zoom camera and processed in CorelDraw.

Histology and immunohistochemistry

Knee joints were isolated at euthanasia, fixated in 4% formaldehyde in PBS pH 7 and subsequently decalcified in 15% EDTA, pH 7.4. Joints were paraffin embedded and cut into 5 μm sections. After deparaffinization and hydration, the sections were blocked for non-specific binding in TBS containing 0.5% casein (TBS/CAS) and subsequently incubated with mouse monoclonal anti-CTX-II antibody (Nordic Bioscience) diluted in TBS/CAS. Peroxidase-labelled rabbit anti-mouse Envision (DAKO Cytomation, Denmark) was used as secondary antibody. Immunoreactivity was visualized by liquid DAB+ substrate chromogen solution (Sigma-Aldrich), and sections were washed in tap water and counterstained with Ehrlich's haematoxylin. Digital images were obtained using an Olympus BX-60 microscope and an Olympus C5050 zoom camera, and processed in CorelDraw. Specificity of the CTX-II immunostaining has previously been demonstrated by staining with an isotype-matched murine antibody and peptide competition (Oestergaard et al. 2006a, Sondergaard et al. 2006a).

Statistics

Results shown are mean ± SEM. Differences between means of ACLT and sham-operated knees were compared using the Student's two-tailed unpaired t-test. Differences were considered statistically significant if $p < 0.05$.

Results

Rats tolerated the surgery well and did not show any altered mobility due to surgery during the observation period. Weights of the animals were not affected by the ACLT surgery (data not shown).

By non-enzymatic extraction of proteins from the knee joints, we were able to quantify the concentration of type I and II collagen degradation products within whole ACLT- or sham-operated knee joints. We observed an increase in CTX-II concentration in ACLT joint extracts in comparison with sham-operated joints on day 7 (36.6 ± 13.4 vs. 84.2 ± 25.5 pg ml^{-1}; +230%, $p = 0.07$), day 14 (46.8 ± 16.8 vs. 142.3 ± 32.8 pg ml^{-1}; +304%, $p = 0.04$), and on day 28 (20.0 ± 7.3 vs. 48.4 ± 7.8 pg ml^{-1}; +242%, $p = 0.04$) (Figure 1A). In contrast, the concentration of hydroxyproline in joint extracts (Figure 1B) showed no significant differences between groups at any time point (range 101.3 ± 13.4 to 128.5 ± 10.2 μg ml^{-1}). When correlating CTX-II to

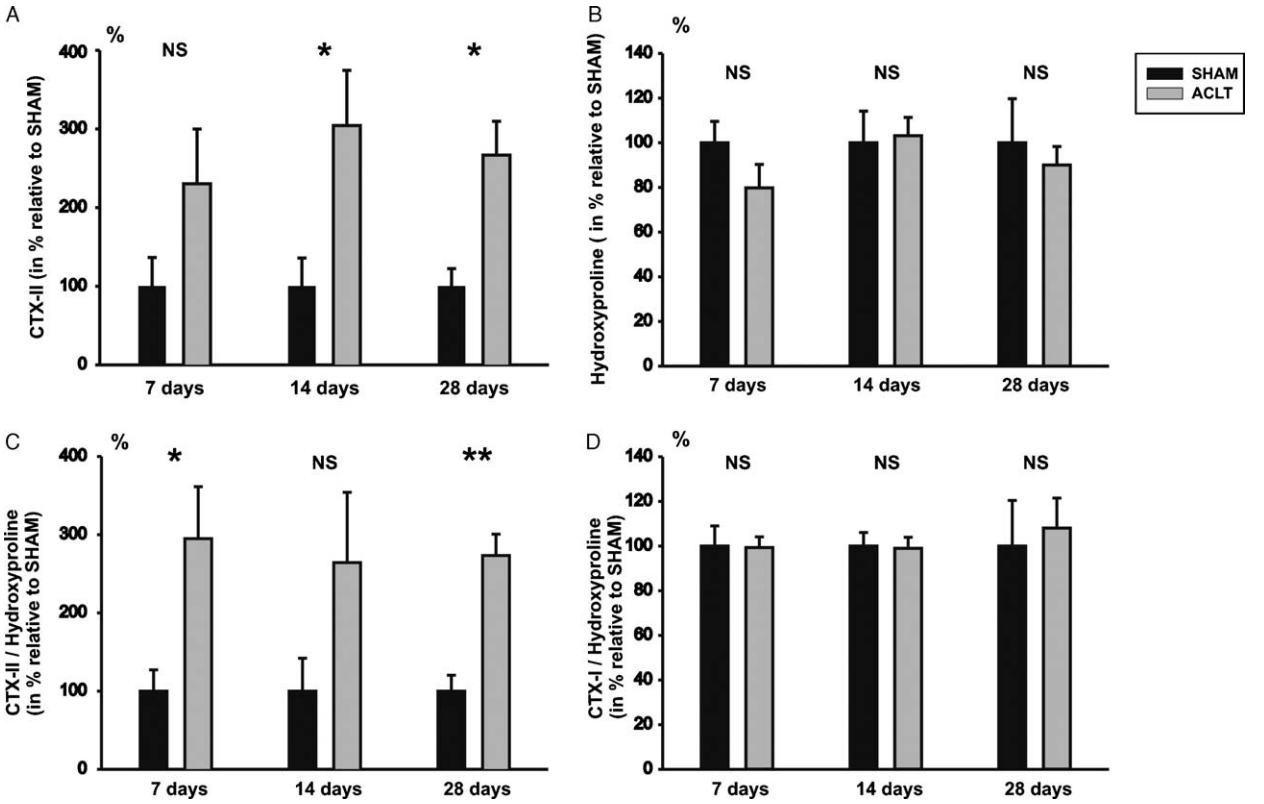

Figure 1. Measurements of (A) C-terminal telopeptide of type II collagen (CTX-II), (B) hydroxyproline, (C) CTX-II/hydroxyproline and (D) CTX-I/hydroxyproline in protein extracts from knee joints which underwent an anterior cruciate ligament transection (ACLT) or a sham operation. Results are presented as mean+SEM in percentage of sham-operated knees. Each bar represents four samples. Statistical analysis: t-test; NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$.

total hydroxyproline (Figure 1C) the CTX-II/hydroxyproline ratio was increased to 294% on day 7 ($p = 0.03$), 264% on day 14 ($p = 0.15$) and 273% on day 28 ($p = 0.002$), compared with sham-operated knees. Neither the absolute concentration of CTX-I (range 30.2 ± 4.5 to 46.2 ± 3.2 pg ml^{-1}, data not shown) or CTX-I correlated to hydroxyproline (Figure 1D) showed any significant difference between groups.

Joint extracts were also investigated for MMP activity by gelatinase zymography. A slight but notable increase in MMP-9 and MMP-2 activity was observed in joints subjected to the ACLT operation compared to sham-operated joints (Figure 2A).

To confirm that the increase in local CTX-II levels was due to cartilage degradation, paraffin-embedded sections were stained for the presence of CTX-II epitopes (Figure 2B). In sham-operated knees CTX-II staining was present in the articular cartilage. In ACLT-operated knees, a strong increase in type II collagen degradation was visible, which co-localized with superficial erosions and loss of proteoglycans in the medial compartment of the tibial joint surface. These structural alterations were present in knees subjected to ACLT both 14 and 28 days post-surgery.

Discussion

The present study assessed the utility of collagen markers in simple protein extracts from joints as a quantitative measure of cartilage and bone status.

When comparing levels of degradation products in protein extracts from ACLT- and sham-operated knee joints, we found no differences in hydroxyproline or CTX-I concentration between joints isolated 7, 14 or 28 days after ACLT operation, suggesting that bone resorption and bone pathology were not altered during the observation period. These biochemical findings are supported by our histological observation that did not reveal any changes in the subchondral bone or presence of osteophytes.

In contrast, we observed elevated concentrations of CTX-II in ACLT-operated knees isolated 7, 14 and 28 days after surgery, indicating an increased type II collagen degradation. Histological evaluation revealed the local presence of CTX-II epitopes co-localizing with surface erosions of articular cartilage, also indicated by loss of proteoglycans in the anatomical region. Previous investigations by Stoop et al. (2001) using the same timeframes but another type of collagen type II degradation marker (C2C) revealed similar findings, also reported by other investigations (Hayami et al. 2006). In addition, the presence of CTX-II epitopes in conjunction with structural alterations of articular cartilage was demonstrated in other experimental models of OA, such as in the ovariectomized rat (Hoegh-Andersen et al. 2004, Oestergaard et al. 2006b). The increased type II collagen degradation in ACLT-operated knee joints could potentially be detected in systemic fluids, as the CTX-II epitope is released into the circulation (Sondergaard et al. 2006b). However, levels of biomarkers released

Figure 2. (A) Representative example of matrix metalloproteinase (MMP) activity in knee joints that underwent a sham or an anterior cruciate ligament transection (ACLT) operation. Zymography indicated a slight but notable increase in enzyme activity in extracts from knees that underwent ACLT. (B) Immuno- and toluidine-blue staining for the tissue presence of C-terminal telopeptide of type II collagen (CTX-II) epitopes (left panel) and proteoglycan (PG) content (right panel) in histological sections from knee joints that underwent a sham or ACLT operation 14 or 28 days prior to termination. Images are obtained from the medial part on the tibial chondyle. The dark staining shows the presence of type II collagen degradation products, i.e. CTX-II epitopes (left panels). Absence of purple colour indicates loss of proteoglycans (right panels).

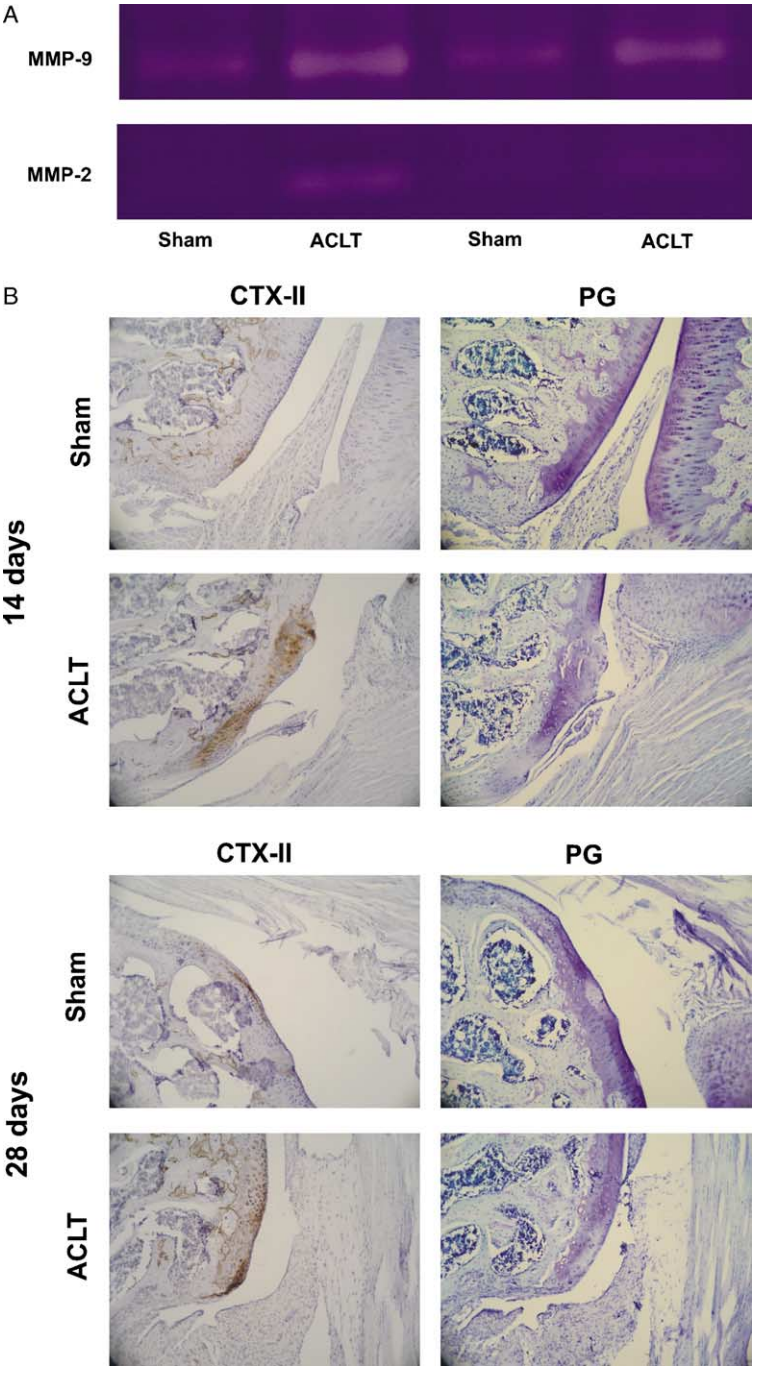

Figure 2 (Continued)

from a single inflicted joint are most likely too low to be, distinguished from normal physiological turnover. The present experiment was not designed for measurements in serum as all animals were subjected to one ACLT operation in one knee and a sham operation in the contralateral knee.

Previous in vitro experiments have demonstrated that MMPs play an essential role in the generation of CTX-II epitopes (Sondergaard et al. 2006a). Using gelatinase zymography, we therefore investigated whether the tissue activity of these enzymes was increased. Indeed, we found a small increase in MMP-2 and MMP-9 activity in the protein extracts of knees subjected to ACLT, which is in line with previous reports demonstrating increased MMP activity in articular cartilage harvested from different animal models of OA (Flannelly et al. 2002, Hayami et al. 2004) and patients with OA (Lark et al. 1997). This probably explains part of the increased levels of CTX-II found in protein extracts from ACLT knees, as increased MMP activity results in elevated collagen type II degradation (Kozaci et al. 1997, Sondergaard et al. 2006a). However, the moderate increase in MMP-2 and MMP-9 suggests that other MMPs might be involved. Collectively, the underlying mechanisms promoting collagen type II degradation and concomitant structural damage involve activation of MMPs, although the exact profile of these remains to be determined.

In summary, our data show that quantitative measures of bone and cartilage degradation epitopes in protein extracts from joints can provide useful information about joint pathology in a compartment specific manner.

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