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Relative contribution of matrix metalloprotease and cysteine protease activities to cytokine-stimulated articular cartilage degradation

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Summary

Objective: Both matrix metalloprotease (MMP) activity and cathepsin K (CK) activity have been implicated in cartilage turnover. We investigated the relative contribution of MMP activity and CK activity in cartilage degradation using *ex vivo* and *in vivo* models.

Methods: Bovine articular cartilage explants were stimulated with oncostatin M (OSM) 10 ng/ml and tumor necrosis factor-α (TNF-α) 20 ng/ml in the presence or absence of the broad-spectrum MMP inhibitor GM6001 and the cysteine protease inhibitor, E64. Cartilage degradation was evaluated in the conditioned medium by glycosaminoglycans (GAG), hydroxyproline, and cross-linked C-telopeptide fragments of type II collagen (CTX-II), which were compared to immunohistochemical evaluations of proteoglycans and CTX-II. We assessed MMP expression by gelatine zymography and CK expression by immunohistochemistry. *In vivo*, CTX-II release was measured from CK-deficient mice.

Results: OSM and TNF-α combined induced significant ($P < 0.01$) increase in cartilage degradation products measured by hydroxyproline and CTX-II compared to vehicle control. The cytokines potently induced MMP expression, assessed by zymography, and CK expression investigated by immunohistochemistry. Inhibition of MMP activity completely abrogated hydroxyproline and CTX-II release ($P < 0.01$) and GAG release ($P < 0.05$). In contrast, E64 resulted in increased CTX-II release by 100% ($P < 0.05$) and inhibited GAG release by 30%. Up-regulation of CTX-II fragments was confirmed *in vivo* in CK null mice.

Conclusion: Inhibition of MMP activity reduced both proteoglycan loss and type II collagen degradation. In contrast, inhibition of cysteine proteases resulted in an increase rather than a decrease in MMP derived fragments of collagen type II degradation, CTX-II, suggesting altered collagen metabolism.
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Key words: Articular cartilage, Collagen type II fragments, CTX-II, TNF-α, Oncostatin M, Matrix metalloproteases, Cathepsin K.

Introduction

Osteoarthritis (OA) is a major cause of functional impairment and disability among the elderly[1], however, current therapies predominantly alleviate symptoms rather than protect cartilage. Better understanding of the mechanisms of cartilage degradation is essential for identifying targets, which promise effective prevention and treatment. Protease activity has been a target for intervention in OA to reduce degradation of cartilage[2]. However, compensation by other proteases has been suggested to attenuate efficacy[3], which potentially could lead to deleterious side effects. This warrants further investigation of the interrelationships between various families of proteases.

The major part of cartilage is composed of collagen type II (60–70% of dry weight) and proteoglycans (10% of dry weight) of which aggrecan is the most abundant[4]. The key mediators of cartilage degradation *in vivo* and *in vitro* include the matrix metalloproteases (MMPs) and members of the closely related family of a disintegrin and metalloproteases (ADAMs) with thrombospondin motifs (ADAM-TS)[5,6]. Aggrecan is degraded both by MMPs and ADAM-TSs, in particular ADAM-TS4 and ADAM-TS5[7–11], whereas collagen type II for the major part is degraded by various MMPs[12,13]. The action of these endopeptidases in articular cartilage results in the release of various extracellular fragments from the articular matrix, of which aggrecan and collagen type II are primarily investigated[14,15]. However, recent evidence has suggested a role of the cysteine protease, cathepsin K (CK), in addition to the well-established role of MMPs in cartilage degradation[16,17]. CK was shown to degrade aggrecan both at acidic and neutral pH as well as collagen type II at acidic pH[18–22] and to a lower extent at neutral pH[20]. With these observations in mind, CK was speculated to be a pharmaceutical target for degenerative cartilage diseases[19,20,23].

Several studies have provided evidence that oncostatin M (OSM) and tumor necrosis factor-α (TNF-α) are inducers of cartilage degradation *in vitro* and *in vivo*[13]. OSM is

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a member of the interleukin-6 (IL-6) family that has previously been shown to have synergism with a number of pro-inflammatory cytokines in promoting collagen degradation[13,24–26]. Recently, OSM and TNF-α were shown to be potent and synergistic inducers of cartilage degradation, by induction of MMP-1, 3, and 13[13].

We wanted to investigate the proteases involved in cartilage degradation and to investigate in more detail the proteolytic machinery responsible for the cartilage catabolic activity in response to OSM and TNF-α. The aim of the present study was to use an *ex vivo* articular cartilage culture system to investigate the relative contribution of MMP activity and cysteine proteinases in cartilage turnover. We investigated collagen type II turnover by measuring hydroxyproline and neoepitopes from collagen type II degradation (CTX-II)[27–29], and proteoglycan turnover by toluidine blue staining and measurements of glycosaminoglycan (GAG) release in the conditioned medium.

Materials and methods

REAGENTS

The reagents applied were of analytical grade. The culture medium comprised Dulbecco's Modified Eagle Medium (D-MEM) with penicillin and streptomycin, all from Life Technologies, US. Human recombinant OSM was obtained from Sigma–Aldrich, UK, and human recombinant TNF-α, from R&D systems, UK. The broad-spectrum ADAM/MMP inhibitor[30–32] *N*-[2(*R*)-2-(hydroxamido carbonylmethyl)-4-methylypentanoyl]-L-tryptophane methylamide, GM6001, was supplied from AMS Scientific, US, the cysteine protease inhibitor, E64, and the synthetic membrane permeable analogue, E64d, were from Sigma–Aldrich, UK.

TISSUE PREPARATION

Bovine articular cartilage explants were carefully harvested by scalpelling the outermost layer of articular cartilage without adherent calcified cartilage from bovine heifer stifle joints between 1 and 1.5 years. The cartilage explants (16 ± 4 mg) were washed three times in PBS and placed in 96 well plates and incubated at 37 °C, 5% CO_2 with shaking at 50 rpm, and cultured under serum-free conditions in 200 μL D-MEM containing cytokines, as specified in the figure legends. As control, articular cartilage explants and metabolic inactivated explants were cultured in D-MEM. In order to deactivate the metabolism of the articular cartilage explants used for "metabolic inactive" treatment, the explants were placed in cryotubes (Nunc, DK), then frozen in liquid N_2 and thawed at 37 °C in water-bath for three repeated freeze-thaw cycles. The conditioned medium was replaced every 3rd day with freshly prepared medium. The supernatants were stored at −20 °C until further analysis.

BREEDING OF MICE

CK-deficient mice were generated as previously described[33] and outcrossed to the C57BL/6 background. F2-generation from intercrossing with normal CK genotype was used for the experiments. Thirteen CK−/− and 17 CK+/− mice were sacrificed at week 10 after birth and blood samples were collected for investigation of CTX-II levels.

GENOTYPING OF MICE

Tail tips of each investigated mouse of F2-generation were taken and digested in lysis buffer overnight to determine the genotype[33]. The disruption of the CK-gene was tested by polymerase chain reaction (PCR) using primer A "5′-GCC-ACA-CCC-ACA-CCC-TAG-AAG-3′" and primer B "5′-ACA-AGT-GTA-CAT-TCC-CGT-ACC-3′". Mice, that were heterozygous for CK, displayed two specific PCR bands at 1.6 kb and 0.4 kb. CK-deficient mice only showed the PCR band at 1.6 kb.

MEASUREMENTS OF HYDROXYPROLINE

Collagen degradation was evaluated by measuring the content of hydroxyproline in the explants' supernatants using a modified version of Podenphant *et al.*[34]. Briefly, 10 μL sample was diluted five times in 7.5 M HCl and hydrolyzed 20 h at 110 °C, and the liquid was evaporated overnight at 50 °C. The precipitate was dissolved in 250 μL isopropanol milli Q water solution (2:1). Then 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 4 ± 1 min at room temperature and added 130 μL of reagent B (reagent B (3:13) of 4.5 M 4-dimethylamino-benzaldehyde dissolved in 60% perchloric acid and isopropanol). The samples were incubated 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. Bone samples digested with collagenase were used as controls.

MEASUREMENTS OF GAG

For detection of sulfated glycosaminoglycans (sGAG), the quantitative dye-binding assay for *in vitro* analysis of GAG release was used according to the manufacturer's instructions (Wieslab, S). In short, samples, standards, blank, and control were added to guanidine–HCl and incubated for 15 min, 0.3% H_2SO_4 and 0.75% Triton X-100 was added and the samples were incubated for 15 min. Finally Alcian blue working solution was added followed by 15 min incubation. The samples and standards were centrifuged for another 15 min and the pellets were dissolved in Dimethyl Sulfoxid (DMSO), and the solutions were centrifuged again at 12,000*g* and then dissolved in 4 M guanidine–HCl, 33.3% 1-propanol and 0.25% Triton X-100. The absorbance was read at 620 nm on an ELISA-reader.

BIOCHEMICAL MARKERS OF CARTILAGE DEGRADATION

Conditioned medium

C-telopeptide fragments of type II collagen were measured in the pre-clinical (PC) CartiLaps ELISA (Nordic Bioscience Diagnostics, DK); an enzyme-linked immunosorbent assay (ELISA) based on mouse monoclonal antibody targeting a six amino acid epitope (EKGPDP) at the C-terminal telopeptide of collagen type II. The PC CartiLaps ELISA is a modification of the Urine CartiLaps ELISA[27,35] optimized for the detection of CTX-II fragments in animal urine and culture medium. This assay has no significant cross-reactivity with type I collagen C-telopeptides[27,35]. The detection limit of the assay is 0.75 ng/ml and the intra- and inter-assay coefficients of variation (CV) in the PC CartiLaps assay are lower than 6%.

Serum

Serum PC CTX-II sandwich ELISA was used for the estimation of cartilage degradation in serum samples obtained from rodents (Nordic Bioscience Diagnostics, DK). The assay is based on highly specific monoclonal antibodies raised against the amino acid sequence EKGPDP located at the C-terminal telopeptide of collagen type II. The lowest detection limit is 1.1 pg/mL and the intra- and inter-assay CV is below 8.0%.

ENZYMATIC ASSAYS FOR COLLAGEN TYPE II DEGRADATION

All reactions were carried out in 0.5 mL of MMP buffer (50 mM Tris–HCl buffer (pH 7.5), 10 mM $CaCl_2$, and 1 mM $ZnCl_2$). Bovine articular cartilage (2–3 mg) was submerged in liquid nitrogen and defrosted three times. Then the cartilage was washed three times in MMP buffer. MMP-9 and MMP-13 (R&D systems, US) were APMA (*p*-aminophenylmercuric acetate) activated by incubation with 1 mM APMA for 2 h at 37°C. After activation, 10 μL of enzyme suspension was added to 0.490 mL aliquots of bovine cartilage explants resulting in a final concentration of 20 nM MMP-9 or MMP-13. Incubations were performed in triplicates for 24 h at 37°C under gentle agitation. The reaction was stopped by adding 10 μL of 10 mM GM6001 (AMS Scientific, US). The mixture was centrifuged for 20 min at 1500*g* at 4°C, and the supernatants were kept frozen at −80°C before measurement for CTX-II. Cartilage alone, cartilage + MMP, MMP buffer alone (as negative control), and cartilage + MMP + GM6001 were measured in the CartiLaps ELISA (Nordic Bioscience Diagnostics, DK).

ZYMOGRAPHY

MMP expression and activity were determined by gelatinase zymography using 0.5 mg/mL of gelatine (Sigma–Aldrich, UK) as a substrate in 7.5% SDS-polyacrylamide gels. A volume of 5 μL of culture supernatant, rainbow marker (Amersham, US), and gelatinase zymography standards for both MMP-2 and MMP-9 (Chemicon, US) and MMP-2 alone (Amersham, US) were loaded on the zymography gels, and the proteins were separated. After electrophoresis the gels were washed three times with 2.5% Triton X-100 in water and then incubated overnight at 37°C in 0.1% Triton X-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, UK) in 10% acetic acid and 45% methanol and destained for 30 min with 20% acetic acid, 20% methanol, 17% ethanol, 0.6% diethylether. Gels were dried and scanned for documentation.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

The bovine articular cartilage explants were fixed in 3.7% formaldehyde in PBS pH 7, paraffin embedded, and subsequently sectioned into 5 μm sections. The proteoglycans were stained by toluidine blue after deparaffinization and rehydration. Immunohistochemistry was performed using monoclonal mouse antibodies against CK (Chemicon, US) and CTX-II (Nordic Bioscience Diagnostics, DK). The sections used for immunolocalization of CK were pre-treated in 10 mM Tris and 0.25 mM Ethylenediaminetetraacetic acid (EDTA), pH 9 overnight to enhance epitope availability. CTX-II immunolocalization was performed without pre-treatment of the sections. The cartilage sections were blocked for non-specific binding in TBS containing 0.5% casein (TBS/CAS) and subsequently incubated with mouse monoclonal CTX-II antibody diluted in TBS/CAS. Peroxidase labeled mouse Envision (DAKOcytomation, DK) was used as secondary antibody. Finally, the immunoreactivity was visualized by liquid DAB + substrate chromogen solution (3,3′-diaminobenzidine chromogen solution; Sigma–Aldrich, UK) followed by rinsing of the slides in tap water. As control, the CartiLaps-peptide (Pepceuticals Ltd., UK) was used in 50-M excess to evaluate the specificity of the CTX-II staining. A matching IgG1κ was used as negative control parallel to the anti-CK. The sections were counterstained with Ehrlich's hematoxylin. Digital histographs were taken using an Olympus BX-60 microscope and an Olympus C5050 zoom camera.

STATISTICS

All graphs show one representative experiment of at least four, each with at least four individual replications. Mean values and S.E.M. were calculated using GraphPad Prism, and compared by the Student's two-tailed unpaired *t*-test of statistical significance assuming normal distribution where four replications were used. The *asterisks* indicate the significance levels ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Results

COLLAGEN TYPE II DEGRADATION IS INDUCED BY OSM AND TNF-α

Combined stimulation of bovine articular cartilage explants by OSM and TNF-α evoked marked increases in cartilage degradation. The conditioning medium was fully replaced every 3rd day, freshly prepared with cytokines. TNF-α (16 ng/ml) in presence of OSM (10 ng/ml) time-dependently induced cartilage degradation as measured by CTX-II [Fig. 1(A)].

TNF-α concentration-dependently increased the release of CTX-II compared to the controls [Fig. 1(B)], and the highest concentration of TNF-α (64 ng/ml) gave more than 1000-fold increase in CTX-II release. Prolonged exposure of explants to this combination of OSM and TNF-α led to complete digestion of the explants after 31 days of culture (data not shown). The magnitude of increase in CTX-II release measured after 28 days of incubation from OSM (10 ng/ml) and TNF-α (16 ng/ml) was 500-fold compared to untreated vehicle controls and 1000-fold compared to metabolically inactivated cartilage explants [Fig. 1(B)]. The cytokines alone in the tested concentrations did not induce cartilage degradation as measured by CTX-II release even after prolonged culture (data not shown). Thus, a synergistic effect of the cytokines was observed from 10 ng/ml OSM and 4 ng/ml TNF-α. From these experiments, the culture conditions were decided to be 10 ng/ml OSM and 20 ng/ml TNF-α, which allow investigation of both inhibitory and stimulatory effects on cartilage turnover.

OSM AND TNF-α INDUCE MMP EXPRESSION

To investigate the proteases responsible for the degradation of articular cartilage, we investigated the expression of gelatinases by gelatine zymography[36,37]. Conditioned medium from six time-points, control and the corresponding cytokine-stimulated explants were loaded on the gels. As shown in Fig. 2, we found a low expression of gelatinases in the absence of cytokines. However, in the presence of OSM and TNF-α we identified high expression of bands corresponding to MMP-9 and MMP-2, as determined by their

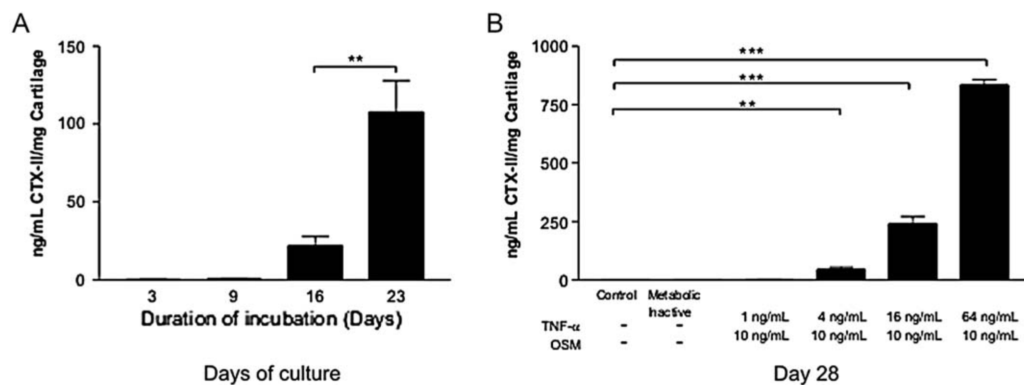

Fig. 1. TNF-α induces time and concentration-dependent cartilage degradation. Bovine articular cartilage explants were cultured under serum-free conditions with a combination of the cytokines OSM and TNF-α for 23 days. Every 3rd day the conditioning medium was fully replaced with fresh medium with or without cytokines as specified under the figures. (A) CTX-II release from days 3, 9, 16, and 23 from articular cartilage explants cultured in the presence of OSM 10 ng/ml and TNF-α 16 ng/ml. (B) CTX-II release in the absence or presence of increasing concentrations of TNF-α at day 28 of culture. The CTX-II release is adjusted for the amount of cultured cartilage. Each bar represents the mean value + S.E.M. from four individual wells. The *asterisks* indicate significant differences ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

corresponding standards, and to a lower extent other gelatinases as well. At late time-points, day 13 and day 17, a band appeared with an apparent molecular weight of 40 kDa corresponding to active MMP-13 activity, as previously observed by zymography[38]. In parallel, as a control for MMP activity, the gelatine zymography gels were incubated with the general MMP inhibitor GM6001 (50 μM)[39], and the gelatinase activity was completely abrogated (data not shown).

MMP-9 AND MMP-13 DEGRADE COLLAGEN TYPE II RESULTING IN CTX-II EPITOPES

Previous investigations have strongly implicated MMP-9 and MMP-13 in several cartilage pathologies[40], and the present studies show that in particular MMP-9 activity was induced in the articular cartilage cultures stimulated with OSM and TNF-α to induce cartilage degradation. Thus, we investigated the ability of MMP-9 and MMP-13 to generate CTX-II epitope. As presented in Fig. 3, incubation of native articular cartilage with 20 nM of MMP-9 or MMP-13 resulted in a 100-fold increase in collagen degradation, as measured by CTX-II. GM6001 was used as control and completely abrogated collagen type II degradation, as measured by CTX-II, demonstrating that this collagen degradation was MMP specific.

OSM AND TNF-α INDUCE CK EXPRESSION

To further investigate the protease machinery responsible for the generation of the CTX-II epitope, we investigated CK expression in both the OSM and TNF-α stimulated cartilage explants and the control articular cartilage explants, using immunohistochemistry. In the absence of OSM and TNF-α we were unable to detect CK expression [Fig. 4(A)]. However, in the presence of OSM and TNF-α high immunoreactivity of CK was detected [Fig. 4(B)].

OSM AND TNF-α INDUCED GAG RELEASE IS STRONGLY ATTENUATED BY INHIBITION OF MMP ACTIVITY AND TO A LESSER EXTENT BY INHIBITION OF CYSTEINE PROTEASE ACTIVITY

To investigate the role of MMPs and cysteine proteases in proteoglycan turnover in the articular cartilage explants' model we measured GAG release induced by OSM and

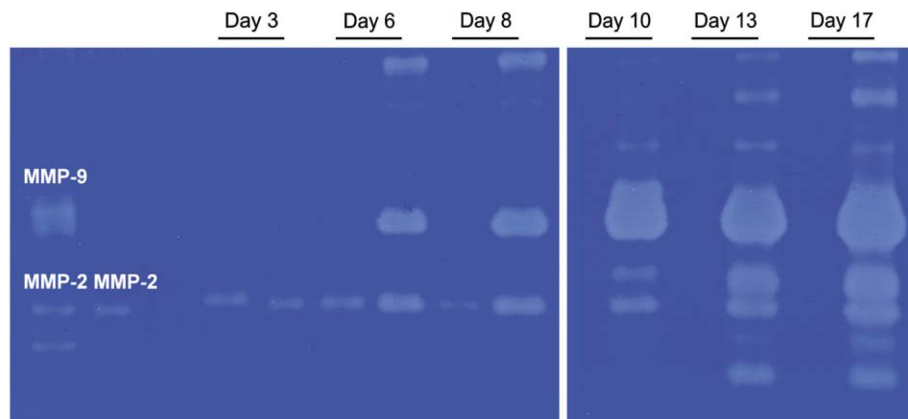

Fig. 2. OSM and TNF-α induce MMP-2 and MMP-9 expression. Gelatinase activity was investigated by zymography in conditioned medium from bovine articular cartilage explants. Standards for MMP-9 + MMP-2 and MMP-2 alone was used. The adjacent lanes, from either 3, 6, 8, 10, 13, or 17 days of culture are conditioned medium without cytokines, and the corresponding OSM 10 ng/ml and TNF-α 20 ng/ml stimulated cartilage. MMP-2 and MMP-9 activities increased over time in conditioned medium from bovine articular cartilage explants cultured with OSM 10 ng/ml and TNF-α 20 ng/ml. Each lane represents individual wells.

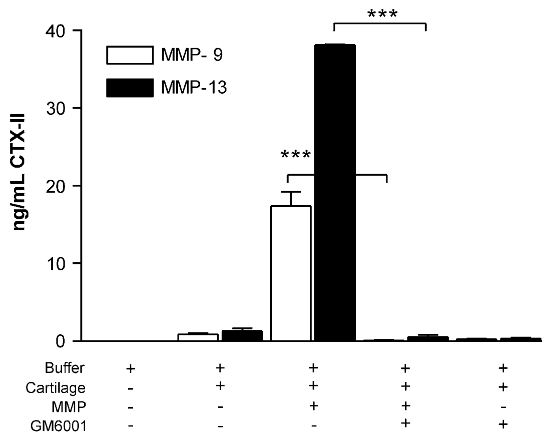

Fig. 3. MMP-9 and MMP-13 induce CTX-II release *in vitro*. Articular cartilage was incubated with or without MMP-9 and MMP-13 in MMP buffer and the general MMP inhibitor, GM6001, as control. The suspensions were centrifuged and cross-linked neoepitopes from type II collagen (CTX-II) were measured from the supernatants. The *asterisks* indicate significant differences (***$P < 0.001$).

TNF-α. To investigate the contribution from the proteases, we used the MMP inhibitor GM6001 and the cysteine protease inhibitor E64 [Fig. 5(A,B)]. OSM and TNF-α induced a more than twofold significant ($P < 0.01$ and $P < 0.001$) increase in GAG release both at days 2 and 6, compared to vehicle treated control as seen in Fig. 5(A and B), respectively. Inhibition of MMP activity by 10 μM GM6001 inhibited the OSM and TNF-α induced proteoglycan release by approximately 70% [Fig. 5(A,B)]. The cysteine protease inhibitor, E64 (40 μM), inhibited the GAG release by 30–40%, albeit not significant. Similar results were obtained by the membrane permeable E64d (data not shown). The potency of E64 was confirmed in human osteoclast cultures according to Ref.[41], and was found to completely abrogate CK activity at 40 μM, as measured by the release of collagen fragments from bone slices (data not shown). These results might indicate the relative importance of the different proteases in cartilage degradation. The cell viability was measured by AlamarBlue, as previously described for three-dimensional cell cultures[39], in order to ensure that the decrease in cartilage degradation was not due to cell toxic effects of E64 and GM6001. None of the inhibitors were found to be cell toxic (data not shown).

OSM AND TNF-α INDUCED COLLAGEN TYPE II DEGRADATION IS ABROGATED BY INHIBITION OF MMP ACTIVITY, BUT NOT CK ACTIVITY

As both CK and members of the MMP family are induced by OSM and TNF-α stimulation (Figs. 2 and 4), we investigated the relative contribution of these proteases to the collagen type II degradation investigated by hydroxyproline and CTX-II release in the conditioned medium by the use of the general MMP inhibitor, GM6001, and the cysteine protease inhibitor, E64. The generation of CTX-II epitopes over days and the accumulated data is seen from Fig. 6(A) and the accumulated hydroxyproline release from Fig. 6(B). OSM and TNF-α induced a more than 100-fold increase in cartilage degradation compared to vehicle control as investigated by the measurement of CTX-II fragments in the conditioned medium. Without OSM and TNF-α cartilage degradation was absent, and comparable to metabolic inactive cartilage. This potent induction of cartilage degradation was completely abrogated by inhibition of MMP activity by GM6001. In contrast, inhibition of cysteine protease activity by E64 resulted in a significant ($P < 0.05$) and more than 100% increase in CTX-II release and small but not significant increase in hydroxyproline release. Similar results were obtained with the membrane permeable inhibitor, E64d, an analogue to E64 (data not shown). E64 did not induce collagen type II degradation in cartilage explants without cytokine stimulation (data not shown).

INHIBITION OF MMP ACTIVITY, BUT NOT CK ACTIVITY, ABROGATES PROTEOGLYCAN LOSS *EX VIVO*

To further investigate the role of MMPs and the cysteine proteases in proteoglycan and collagen turnover in the

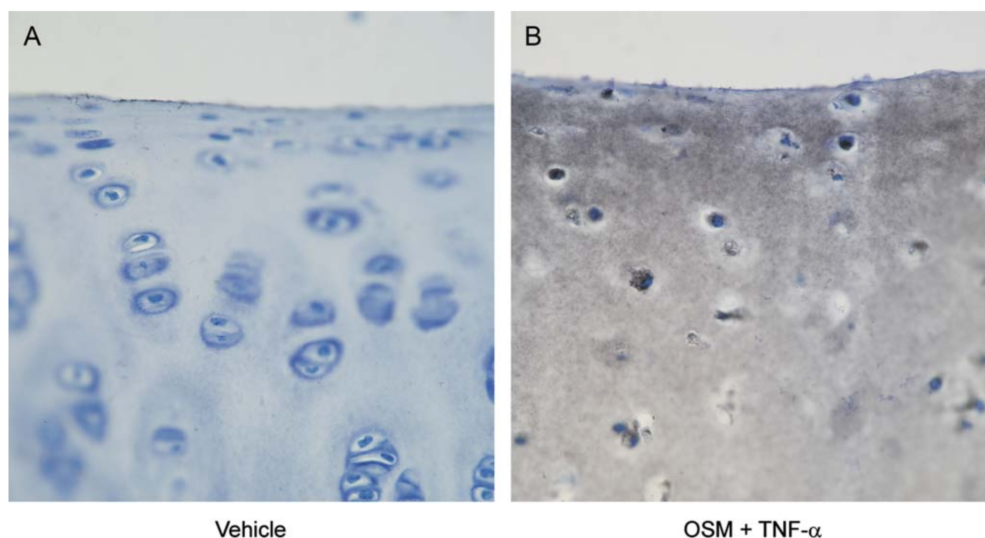

Fig. 4. CK expression is found in OSM and TNF-α activated cartilage. Immunohistochemical analysis of CK in bovine articular cartilage explants. Mouse monoclonal antibody against human CK was used to localize CK in sections from articular cartilage explants. (A) Articular cartilage explants cultured without cytokines. (B) Articular cartilage explants cultured in the presence of OSM 10 ng/ml and TNF-α 20 ng/ml. Tissue sections were counterstained with Ehrlich's hematoxylin.

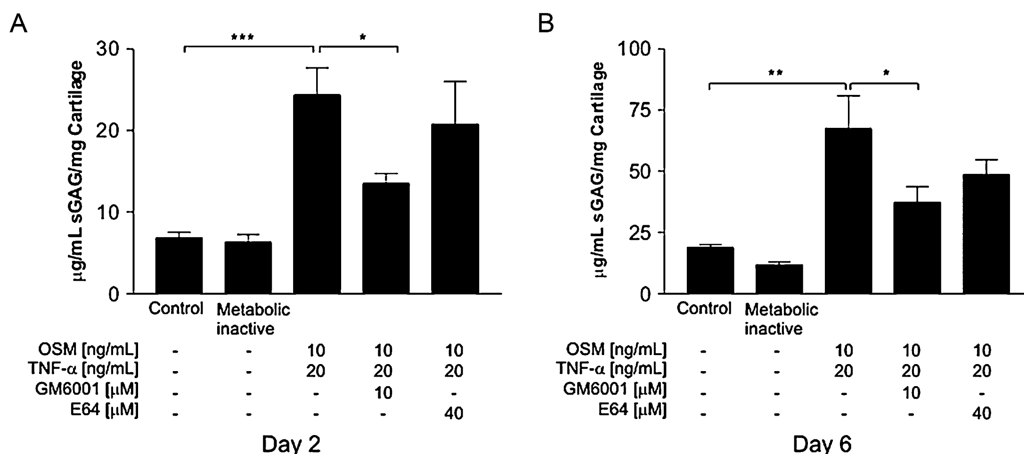

Fig. 5. OSM and TNF-α induced sGAG release was strongly attenuated by inhibition of matrix metalloproteinase (MMP) activity and to a lesser extent by inhibition of cysteine protease activity. Cartilage degradation was induced in bovine articular cartilage explants stimulated with the cytokines OSM 10 ng/ml and TNF-α 20 ng/ml. The conditioned medium was replaced every 3rd day and the quantitative release of sGAG was measured. (A) Represent the sGAG release after 2 days and (B) the sGAG release after 6 days of culture. Each bar represents the mean value + S.E.M. from at least four individual wells, adjusted for the amount of cultured cartilage. GM6001, MMP activity inhibitor; E64, cysteine proteinase inhibitor. The *asterisks* indicate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

articular explants' model we assessed proteoglycan content by histology using toluidine blue staining and CTX-II by using immunohistochemical localization of the CTX-II neoepitope. As presented in the lower panel of Fig. 7 we found very low or absent CTX-II staining in the absence of OSM and TNF-α, which correlate to the presented CTX-II measurements in the conditioned medium shown in Figs. 1(B) and 6(B). OSM and TNF-α stimulated cartilage showed immunolocalization of CTX-II epitope, which is in alignment with the CTX-II measurements. Loss of proteoglycans was visualized by toluidine blue staining (upper panel of Fig. 7) in the extracellular matrix. In line with the measurement of CTX-II in the conditioned medium shown in Fig. 5, inhibition of MMP activity by GM6001 abrogated both proteoglycan loss and collagen type II degradation. In contrast, inhibition of CK activity by the cysteine protease inhibitor, E64, only to a minor extent attenuated the loss of proteoglycans and did not neutralize generation of the CTX-II neoepitope.

COLLAGEN TYPE II FRAGMENTS ARE INCREASED IN CK-DEFICIENT MICE

The *ex vivo* experiments demonstrated significantly elevated CTX-II levels in the absence of CK activity. To further investigate the specific loss of CK activity on generation of CTX-II epitopes *in vivo*, we used serum from 10-week-old mice of either CK-deficient mice[33] or control littermates. Measurements of CTX-II levels, showed 40% significant ($P < 0.01$) increase in CK null mice compared to wild type control, as shown in Fig. 8.

Discussion

We have investigated the relative contribution of MMP and cysteine protease activity in cartilage degradation. We demonstrated that stimulation of articular cartilage explants by OSM and TNF-α resulted in time- and

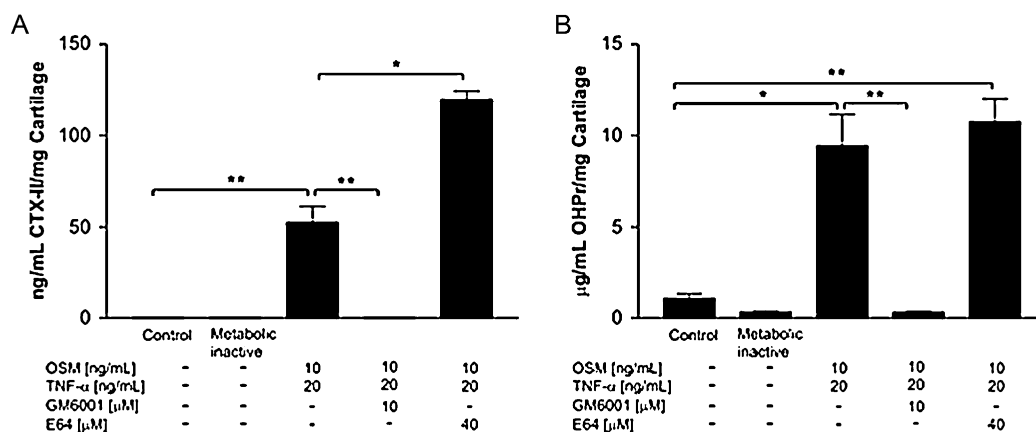

Fig. 6. Collagen degradation is mediated by MMPs, and not cysteine proteases. Bovine articular cartilage explants were cultured under serum-free conditions in the presence of either vehicle or a combination of the cytokines OSM 10 ng/ml and TNF-α 20 ng/ml. The conditioned medium was replaced every 3rd day and the CTX-II release (A) and the hydroxyproline (OHpr) release (B) were accumulated from day 3 to day 27. The CTX-II and the hydroxyproline release were adjusted for the amount of cultured cartilage. Each bar represents the mean value + S.E.M. from four individual wells. GM6001, MMP activity inhibitor; E64, cysteine proteinase inhibitor. The *asterisks* indicate significant differences (*$P < 0.05$, ***$P < 0.001$).

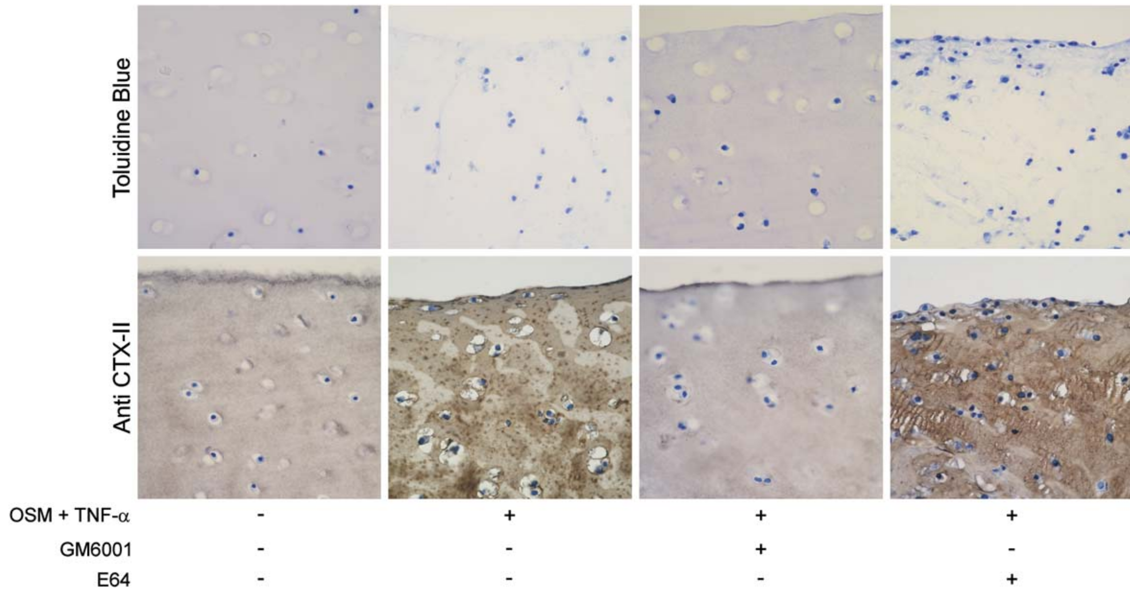

Fig. 7. Immunohistochemical localization of CTX-II fragments generated by MMPs. Bovine articular cartilage explants were cultured under serum-free conditions with or without a combination of OSM + TNF-α, and protease inhibitors where indicated; the general MMP inhibitor GM6001 and cysteine protease inhibitor E64. At the last day in culture, day 23, the explants were fixed in formaldehyde, paraffin embedded and sectioned. The upper panel shows proteoglycan staining by toluidine blue, and the lower panel shows immunohistochemical detection of collagen type II degradation, with a mouse monoclonal antibody against cross-linked C-telopeptide fragments of type II collagen (CTX-II). The tissue sections were counterstained with Ehrlich's hematoxylin.

concentration-dependent cartilage degradation, which was dependent on MMPs and not cysteine protease activity. In addition, we present data indicating that in the absence of CK, or by inhibition of cysteine protease activity, chondrocyte mediated degradation of the extracellular matrix shifts to a more MMP like phenotype, resulting in more MMP generated fragments of collagen type II.

Stimulation with OSM + TNF-α resulted in proteoglycan loss in the initial culture period until day 9, followed by collagen type II degradation starting from day 14. OSM and TNF-α strongly induced CK expression and MMP expression, in

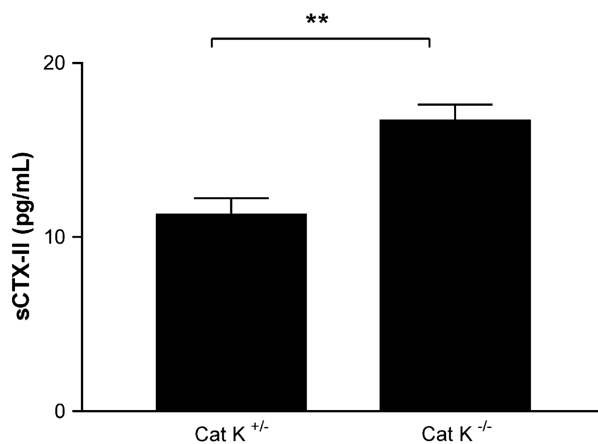

Fig. 8. *In vivo* loss of CK activity leads to increased CTX-II levels. Serum CTX-II measurements were performed on either 10-week-old CK heterozygous or null mutation mice. A total number of 30 mice were used to investigate CK-deficient (CK−/−) in comparison to CK-heterozygous mice (CK+/−) week 10 after birth: CK+/− ($n = 13$) and CK−/− ($n = 17$). The *asterisks* indicate significant differences (**$P < 0.01$).

particular MMP-2 and MMP-9. Inhibition of MMPs by the general MMP inhibitor, GM6001, neutralized the cytokine-stimulated degradation of proteoglycans (GAG) and collagen type II, as measured by hydroxyproline and CTX-II, whereas inhibition of cysteine proteases by E64 and E64d did not restrain the degradation of articular cartilage.

We demonstrated that MMP-9 and MMP-13, which have been implicated in various cartilage pathologies[42], were able to generate CTX-II fragments, and that MMP-13 was more potent than MMP-9 based on equivalent molar concentrations. Gelatinase zymography was used to identify MMP activity from cytokine-stimulated explants, in which primarily MMP-2 and MMP-9 were detected. After prolonged culture a band of approximately 40 kDa was detected that corresponds to the molecular weight of active MMP-13[38], which may be further investigated by casein zymography[43]. MMP-13 is a well-known triple helical protease and was able to generate the CTX-II neoepitope that demands both a C- and N-terminal cleavage to release the peptide from the matrix. Generation of the EKGPDP (CTX-II) neoepitope from the C-terminal part of the collagen type II telopeptide is well described and generated by MMPs, which is demonstrated by the fact that the EKGPDP is a prerequisite for the interaction of the antibody, as intact collagen type II does not cross-react[27,44]. In addition, from these experiments it can be concluded that not only the triple-collagenase MMP-13 but also the gelatinase MMP-9 resulted in CTX-II generation, thus collagen metabolism seems not to be restricted to triple-collagenase enzymes. More research on the exact composition of the N-terminal fragment is needed in order to further understand the collagen type II metabolism of the chondrocytes, as multiple protease sites are found upstream in collagen type II that may allow the release of the CTX-II fragment.

We found that inhibition of MMPs completely abrogated collagen type II degradation, as measured by hydroxyproline

and CTX-II release, and restored proteoglycan loss from the articular cartilage explants to near control levels. Several MMPs have been shown to cleave aggrecan[45–47], albeit ADAM-TS4 and 5 are the most important *in situ* mediators of aggrecan degradation[8,9,48]. Presently, only MMPs have been identified to cleave collagen type II, however, the broad-spectrum MMP inhibitor GM6001[39] may indeed target various ADAM-TS. Tortorella *et al.*[49], have previously described that ADAM-TS4 and 5 were unable to cleave collagen type II, and future studies are required to address the ability of ADAM-TS and MMPs to generate the CTX-II epitope.

Inhibition of cysteine protease activity by E64 and E64d in OSM and TNF-α stimulated cartilage resulted in a small decrease in proteoglycan degradation. Cysteine proteases have been shown to be able to degrade aggrecan[20,50], which suggests a minor role of these proteases in cartilage turnover under diseased conditions. Inhibition of cysteine protease activity in cytokine-stimulated cartilage explants did not attenuate collagen type II degradation, evaluated by hydroxyproline, CTX-II release, and immunohistochemistry. Interestingly, inhibition of cysteine proteases significantly increased the CTX-II levels suggesting altered collagen type II metabolism, which may include compensatory mechanisms of other collagenases in the absence of CK activity. These findings were confirmed in the CK null mutation mice that displayed significantly increased serum CTX-II levels. This is in agreement with the mRNA analysis of the CK null mutation mice, which showed a 300% up-regulation of MMP-13 expression and a 50% up-regulation of MMP-9 expression[3]. These biochemical studies indicate that CK-deficient mice may suffer from early arthritis, however, this remains to be investigated. In humans, CK mutations lead to osteopetrosis, and evidence for increased MMP activity in the lack of CK activity is found in these patients[51,52]. Strongly elevated type I collagen fragments (carboxy-terminal telopeptide of type I collagen, ICTP), which are MMP generated, are found in the absence of CK activity, suggesting compensational roles of MMPs[52–54]. Recently, Henriksen *et al.*[53], demonstrated that osteoclasts in the absence of CK activity shifted into a more MMP catabolic mode which results in more MMP, and not CK, derived fragments of collagen type I. Thus, inhibition of CK and other cysteine proteases might lead to altered composition and release of protease generated fragments of type II collagen, however, these data do not rule out the possibility of a direct effect of CK on de-activating members of the MMP family.

The cysteine protease CK was shown to be expressed in some instances of cartilage pathologies and to be involved in collagen type II degradation[18–21,50]. CK is a lysosomal protease that has a pH optimum at approximately pH 5.5[20,21,50], although activity has been reported in particular against aggrecan at neutral pH[20]. The current experiments were performed under cell culture conditions, which may not provide the same pH environment as reported in advanced stages of cartilage pathologies, such as rheumatoid arthritis and OA[37], which may favor CK activity. However, CK activity was shown to mediate proteoglycan degradation at neutral pH[20,50], even though the protective effect on proteoglycan degradation by inhibition of CK activity was minor compared to the strongly attenuated cartilage destruction observed by inhibition of MMP activity, suggesting that CK is involved in proteoglycan degradation, albeit to a lesser extent than MMP activity.

The immunohistochemistry of CK suggested the presence of both secreted and intracellulary stored (lysosomal) CK, similar to that previously reported for osteoclasts[55,56]. The current culture conditions do not affect intracellular pH in the lysosomes of chondrocytes, meaning that pH conditions (5.5) are optimal for the protease CK. Collagen fragments have previously been shown to be transcytosed and degraded inside the cells[57]. Thus, the current experiments do not suggest a large intracellular role of CK in the chondrocytes for the degradation of collagen type II. The inhibitory effect of cysteine proteases on proteoglycan degradation remains to be further investigated with respect to whether this is a result of intracellular or extracellular activity.

The current experiments relied on many parameters for examination of the cartilage; gross examination of the cartilage explants, biochemical markers, immunohistochemistry and histology, which all pointed towards MMPs, but not cysteine proteases, as the essential proteases in cartilage turnover. In particular, the immunohistochemistry performed on cartilage stimulated with OSM and TNF-α in combination with E64 showed almost complete proteoglycan loss (Fig. 7, upper panel) and extensive collagen type II degradation. However, the current investigation has partly been based on the release of the CTX-II epitope, which we show is not dependent on CK activity in cartilage, and partly on quantification of the released hydroxyproline, which showed similar results. Importantly, other cleavage sites in the collagen type II molecule have been shown to depend on CK activity[18,21,50]. Thus, by investigation of other neoepitopes than CTX-II, such as the col2-3/4 epitope[58], which might demonstrate other disease mechanisms than CTX-II, potential effects of CK inhibition could have been identified. However, the hydroxyproline measurements, which is independent of protease generated neoepitopes, did not suggest major roles of CK in articular collagen type II degradation, as no inhibition was observed.

We used both the cysteine protease inhibitor E64 as well as the cell membrane permeable pro-inhibitor E64d that has previously been shown to inhibit proteoglycan release from IL-1 stimulated bovine nasal septum cartilage[59]. Only a small reduction of proteoglycan degradation was found, albeit not significant, when using the inhibitors. Several experimental parameters may explain these differences, as incubation time, choice of tissue, and cytokines are different. Buttle *et al.*[59], used bovine nasal septum cartilage stimulated with IL-1 for very short time periods in contrast to the present studies involving articular cartilage stimulated with OSM and TNF-α for longer time periods. In line with this, IL-1 can activate cysteine proteases in nasal septum cartilage as described by Buttle *et al.*[59], but IL-1 has been found to be less potent to induce cartilage degradation in articular cartilage[12,60]. The cysteine protease inhibitor, E64, targets CK and other cysteine proteases such as cathepsin B and L that might contribute to matrix destruction of articular cartilage[61]. Cathepsin B was recently shown to generate the CTX-II fragment in an *in vitro* experiment, in contrast to CK[62]. However, as we did not detect an effect in the current experiments on collagen type II degradation and only to a lesser extent on proteoglycan degradation, this cross-reactivity of E64 seems to be of less importance.

Conclusion

We propose this *ex vivo* articular explants' culture system as a valuable tool for the molecular characterization of cartilage turnover, due to the high *in vivo* likeness. Using this model, we found that cytokine induced activation of the cartilage led to MMP and CK expression. Inhibition of MMP activity resulted in complete abrogation of collagen type II

degradation. In contrast, inhibition of cysteine protease activity resulted in an increase rather than a decrease in MMP generated collagen type II fragments of cartilage degradation. This model and these results may be valuable when evaluating possible drugs for cartilage diseases.

Competing interests

BC Sondergaard, S Oestergaard, I Danielsen, C Christiansen, and P Qvist are employees at Nordic Bioscience Diagnostics, and K Henriksen and MA Karsdal are employees at Pharmos Bioscience. P Qvist, MA Karsdal, and C Christiansen hold stock in Nordic Bioscience A/S.

Authors' contributions

P Qvist, C Christiansen and MA Karsdal designed the experimental outlay; BC Sondergaard performed the explants' experiments and analysis of biochemical markers, and the hydroxyproline measurements were performed by I Danielsen. K Henriksen and BC Sondergaard made the immunohistochemistry and histological evaluations. H Wulf made the zymography and MA Karsdal the *in vitro* experiment of MMP cleavage. U Schurigt and R Bräuer performed the animal experiments and S Oestergaard evaluated serum samples. MA Karsdal and BC Sondergaard drafted the manuscript, which was read and approved by all authors.

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