Molecular Bases of Disease:

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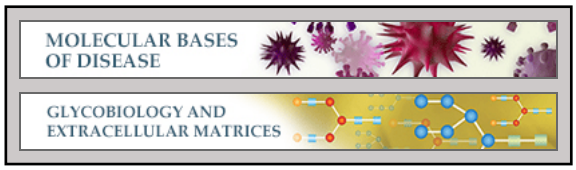

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Proteomic analysis of tendon extracellular matrix reveals disease stage-specific fragmentation and differential cleavage of COMP

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Running title: COMP fragmentation in tendon disease
Keywords: tendon, inflammation, extracellular matrix, mass spectrometry, proteomics, peptides.

Capsule

Background: Tendon disease is characterised by extensive remodelling of the extracellular matrix.

Results: Novel COMP cleavage fragments were identified in both an *in vitro* inflammatory model and natural disease.

Conclusion: Inflammatory mediators drive distinct COMP fragmentation at different stages of tendon disease.

Significance: Novel COMP neo-terminal fragments provide opportunities for developing markers for tendon injury.

Abstract

During inflammatory processes the extracellular matrix (ECM) is extensively remodelled and many of the constituent components are released as proteolytically cleaved fragments. These degradative processes are better documented for inflammatory joint diseases than tendinopathy even though the pathogenesis has many similarities. The aims of this study were to investigate the proteomic composition of injured tendons during early and late disease stages to identify disease-specific cleavage patterns of the ECM protein Cartilage oligomeric matrix protein (COMP). In addition to characterising fragments released in naturally occurring disease, we hypothesised that stimulation of tendon explants with pro-inflammatory mediators *in vitro* would induce fragments of COMP analogous to natural disease. Therefore normal tendon explants were stimulated with IL-1β and PGE_2 and their effects on the release of COMP and its cleavage patterns were characterised. Analyses of injured tendons identified an altered proteomic composition of the ECM at all stages post injury, showing protein fragments that were specific to disease stage. IL-1β enhanced the proteolytic cleavage and release of COMP from tendon explants, whilst PGE_2 had no catabolic effect. Of the cleavage fragments identified in early stage tendon disease, 2 fragments were generated by an IL-1 mediated mechanism. These fragments provide a platform for the development of neo-epitope assays specific to injury stage for tendon disease.

Introduction

Tendons have an abundant extracellular matrix (ECM) and are significant causes of morbidity in athletic individuals (1,2). Repetitive mechanical loading during exercise and inflammation are driving factors in the aetiopathogenesis of injury (3-5). The horse presents an attractive large animal model for the study of the functionally equivalent human Achilles tendon due to the shared characteristics of aging phenotypes (6,7) and elastic energy storing function common to the weight-bearing tendons of both species (8,9). Exercise studies in mature horses have failed to show evidence of an adaptive response to loading and it is suggested that exercise instead drives degeneration, which precedes clinical injury in adult tendon (10,11). This concept was supported by studies in the tensional regions of bovine digital flexor tendons where ECM turnover based on mRNA expression was shown to be low (12) and this has been further supported in recent studies in the horse where the remodelling rate of collagen was found to be negligible (half-life of 198 years) (13). Whilst initial studies of human Achilles tendons suggested continued remodelling in adult tendons based on microdialysis (14,15), recent data has confirmed that the central area of this tendon also has minimal turn-over rate in the adult (16), confirming the horse as a highly relevant model for human tendon disease (17). In contrast, the non-collagenous components of tendon appear to be more rapidly turned over and therefore are likely to be influenced by the degradative processes responsible for the hypothesised changes that occur prior to clinical injury (11,18).

Equine tendon repair processes are frequently clinically classified into three phases in naturally occurring injury; the acute phase occurs immediately after the initial trauma lasting only a few days, followed by sub-acute (3-6 weeks) and chronic injury phases (>3 months after injury) (19). This healing response induces profound changes in the composition of the tendon ECM (20-22) associated with the formation of scar tissue which is believed to be responsible for poor functional outcome in both species (23).

COMP is a pentameric glycoprotein belonging to the thrombospondin family (24) that is found in many mechanically loaded tissues including tendon (25). Its functions are thought to include stabilizing the collagen fiber network and catalyzing fibrillogenesis (26,27), and in assembly, organization and maintenance of the ECM (28). These roles would explain its strong relationship to tendon mechanical properties in equine tendons (29). COMP levels and fragments are elevated in joint disease and reported in the synovial fluids and serum of patients with rheumatoid arthritis and osteoarthritis (26,30). Equids with intrathecal digital flexor tendon tears also show elevated COMP levels in tendon sheath fluids (31,32). COMP degradation is mediated in part by MMPs (33), although MMP independent pathways involving the aggrecanase ADAMTS-4 (28) also occur.

A growing body of recent evidence from studying tendon tissues from humans (34,35), equids (3,36), rodents (37) and *in vitro* models (38) support the role of inflammation in tendinopathy, implicating pro-inflammatory mediators such as IL-1 and PGE_2 in disease development and progression. The role of inflammatory cytokines in non-collagenous matrix breakdown has been investigated extensively in cartilage *in vitro* and *in vivo* as typified by loss of COMP and proteoglycans (28,39-42). While tendon and tendon fibroblasts produce and respond to cytokine stimulation (43), their role in the specific cleavage of ECM proteins is less well documented (28,39,40). The ability to detect specific proteolytic cleavage sites is necessary to understand tendon ECM degradative mechanisms that are disease-stage specific for both targeted therapeutic interventions as well as identifying neo-terminal peptide fragments for developing markers for sub-clinical disease for preventative strategies (41). Equine

tendons present a more readily attainable source than the human counterpart, permitting targeted investigation of disease through each injury phase as well as normal (uninjured) tendons of a wide age range. The aim of this study was to identify COMP fragments generated at different stages of tendon disease and to relate these to those induced specifically by IL-1β and PGE_2 *in vitro*. This is the first comprehensive analysis of tendon ECM degradation in acute and chronic disease and we identify novel COMP fragments in natural disease that are produced by an IL-1β driven mechanism.

Experimental procedures

Collection of equine tendons

Equine forelimbs from Thoroughbred or Thoroughbred cross breed horses aged between 2-20 years were obtained from an abattoir or local equine referral hospital with known history of injury and the tensile (mid-metacarpal) region of the superficial digital flexor tendon (SDFT) harvested within 4 h of death. Tendons were grouped as sub-acutely injured (3-6 weeks post injury, n=6, mean age 9 ± 5 years) or chronically injured (>3 months post injury, n=9, mean age 13 ± 4 years), as described before (3). Tendon injuries were aged based on historical information obtained from either the owner or referring veterinary surgeon prior to euthanasia of the horse. Tendons were classified as normal based on their macroscopic post mortem appearance, which included lack of visible signs of swelling of the tendon body, and a consistent pattern of fascicles on haematoxylin and eosin stained sections (n=19, mean age 8 ± 5 years).

Preparation of tendon explants for tissue culture

Macroscopically normal tendons were used for *in vitro* experiments and derived from horses (n=10) between 7-14 years of age (mean 10 ± 3 years). SDFTs were aseptically dissected from the limb and after removal of the paratenon, tendon explants were cut in a tissue culture flow hood using 3 parallel sterile microtome blades (Surgipath, UK) inserted into a custom designed cutting template to create 2 mm x 2 mm x 37 mm pieces along the longitudinal axis of the tendon (6). Two explants per well were cultured in serum depleted DMEM (3 ml per well) containing 5% penicillin and streptomycin (Invitrogen, UK) in tissue culture 6 well plates (VWR, UK) at 37 °C in humidified atmosphere (5% CO_2 and air). This method of preparing the explant tissue consistently produced average wet weights of 300 mg (±30 mg).

To assess the effects of pro-inflammatory mediators on release of tendon matrix components, explants were stimulated with human recombinant IL-1β (5 $ngml^{-1}$) (Calbiochem, UK) or PGE_2 (0.01 μM or 1.0 μM) (Sigma Aldrich, UK) and release of total collagen and COMP into tissue culture media quantified and compared to non-stimulated controls. After cutting (time 0), explants were incubated in serum deplete media and rested for 24 h to allow the tissue to adapt to the culture environment. Twenty-four hours after explant cutting, media were replaced and samples stimulated with pro-inflammatory mediators. The following inhibitors were added to the experimental system to identify inflammation-relevant release of COMP by intervention of the PGE_2 synthesis pathways (including PGE_2 synthesis via IL-1β): 1.0 μM Firocoxib (Merial, France), 20 μM Ilomastat (Calbiochem, UK), 400 $ngml^{-1}$ recombinant equine IL-1Ra (R&D Systems, UK). Media were harvested and analysed at 48, 72 and 96 h (post-cutting) with complete media replacement at each interval.

Viability of tendon explants in culture

To demonstrate viability of tendon cells at the measured experimental time points, live-dead staining was performed with 4 μM ethidium bromide and 2 μM Calcein AM (Sigma-Aldrich) in PBS containing 5.6 mM glucose, 0.5 mM $MgCl_2$ and 0.9 mM CaCl for 1 h in dark conditions prior to confocal microscopy (Leica Microsystems, Milton Keynes, UK).

Viability of explants cultured in serum deplete DMEM containing 5% penicillin and streptomycin were compared at 24 h and 120 h after cutting, with explants incubated in 2% sodium azide for 24 h as a negative control. ImageJ software (NIH version 1.42) was used to ascertain the proportion of live and dead cells.

Sircol Collagen assay

The Sircol collagen assay (Biocolor Ltd, UK) was used to quantify release of triple helical collagens into tissue culture media as per the manufacturer's instructions. Briefly, 200 µL of culture media was assayed in triplicate in 96-well micro titre plates and the final absorbance read at 555 nm (Sunrise micro plate reader, Tecan, Männedorf, Switzerland). The substrate background absorbance values were subtracted from absorbance readings and a standard curve generated using bovine type I collagen as specified by the assay manufacturer. Results were adjusted to represent collagen release per mg of explant wet weight measured at the termination of the experiment.

COMP ELISA

The COMP ELISA was an in-house assay that has been used successfully with equine samples, the methodology for which is described in detail elsewhere (31,32). COMP release was determined in samples of tissue culture media incubating tendon explants under differing experimental conditions. Results were expressed as µg/ml and subsequently adjusted to represent COMP release per mg of explant wet weight.

SDS/PAGE and Western Blotting of COMP

Western blotting of samples of culture media was used to compare the effects of pro-inflammatory mediators on the release of COMP from tendon. Western blotting of undiluted media was performed under reduced and non-reduced conditions. Samples were reduced by addition of DTT to 0.1 M and heated to 95 °C for 5 mins prior to electrophoresis on 8-10% SDS/PAGE gels. Following electrophoresis, proteins were transferred for Western blotting (Bio-Rad, Hemel Hempstead, UK) onto PVDF membrane (GE Healthcare, Hertfordshire, UK). Membranes were blocked overnight in Tris buffered saline (0.02 M Tris-base, 0.02 M Tris HCl and 0.05 M NaCl) in 1% Triton (TBST buffer) containing 8% powdered skimmed milk (Marvel) and 2% bovine serum albumin (Sigma-Aldrich). After washing in TBST (three times for 10 mins each), membranes were incubated with the COMP primary antibody (25) in a buffer containing 4% (w/v) milk and 1% (w/v) BSA in TBST at a 1:1000 dilution for 2 h. Membranes were washed three times as before and incubated with anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology®, MA, USA) in antibody buffer for 2 h at a 1:2000 dilution. Antibody positive protein bands were visualised using enhanced chemiluminescence (ECL) reagent and film (GE Healthcare). Densitometric analysis of protein bands on non-reduced blots was performed using ImageJ software (NIH version 1.42) using sequential exposures of films to avoid saturation artefacts.

Proteomic analyses using mass spectrometry

Liquid chromatography mass spectrometry (LC-MS) using a Quadruple Time-of-Flight mass spectrometer (Q-TOF) (Q-TOF micro, Waters) were performed on samples of experimental media and extracts of normal and injured SDFTs. Multiple reaction monitoring (MRM) analyses were performed using another LC-MS system comprising of an Easy nano-LC™ (Thermo Scientific) triple quadrupole instrument (TSQ Vantage™, Thermo Scientific) on media samples from tendon explants *in vitro* for one experiment at 72 h, enabling selective quantification of known peptides. The relative costs associated with the use of MS-MS precluded analyses of large numbers of samples. Proteomic analyses using MS-MS were performed to identify ECM proteins and neo-termini of COMP fragments released into media from stimulated

and control normal tendon explants *in vitro,* as COMP was the most abundant ECM protein released from tendon explants in culture. For these proteomic analyses, explants were cut (time 0) and rested for 48 h, as resting for 24 h in pilot studies suggested this was of insufficient duration due to a significant quantity of proteins and peptides released before baseline levels were reached at 48 h. At 48 h, media were replaced and the tissue stimulated with pro-inflammatory mediators for a further 24 h. All media samples for proteomic analyses were analysed at the 72 h time point after explant cutting.

Preparation of media samples for the Q-TOF MS

Care was taken to avoid contamination of samples with skin and hair keratins. 100 µL of media from each sample was reduced with 4 mM DTT and agitated at 56 °C for 30 min. Samples were alkylated with 16 mM iodoacetamide (IAA) at room temperature in the dark for 1 h. Samples were digested with 0.5µg trypsin gold (Promega, Madison, WI) overnight at 37 °C on a shaker for 16 h. Samples were dried in a Speedvac and suspended in 100 µl 0.2% formic acid whereof 10 µl were purified and desalted using homemade reversed phase tips, 4 discs thick (47-mm Empore C18 extraction discs, 3M, Minneapolis, MN) as described before (44,45) and subsequently dried and re-dissolved in 20 µl 0.2% formic acid before injection onto the Q-TOF MS.

Preparation of media samples for the triple quadrupole MS

Media samples (10µl trypsin digest, see above) were cleaned with reversed-phase C18 columns according to the manufacturer's instructions (SEM SS04V-SS18V), columns were purchased from the Nest Group (MA, USA).

Preparation of tendon tissue extracts for QTOF MS analysis

Proteomic analyses were also performed on tissue extracts from macroscopically normal, sub-acute (3-6 weeks post injury) and chronic injured (>3 months post injury) SDFTs to investigate the effect of injury on matrix protein composition. Samples of normal, sub-acute and chronic injured SDFTs (n=3 of each) stored at -80 °C were finely diced and 15 volumes of 4 M GdnHCl containing protease inhibitors (1:100 dilution of protease inhibitor cocktail III, Calbiochem) and 10 mM ethylenediaminetetraacetic acid (EDTA). Samples were rotated at room temperature for 48 h and then centrifuged at 4° C for 15 mins at 13, 000 x g to recover the extract. 50 µL of extract was reduced in 10 mM DTT at 56 °C on a shaker for 30 min and alkylated at 40 mM IAA for 60 min at room temperature in the dark. Extracts were precipitated with ice-cold ethanol (9:1) for overnight at 4 °C before centrifugation (13,200 × g at 4 °C for 30 mins) followed by ethanol wash for 4 h at -20 °C to remove residual GdnHCl and other salts. Samples were dried in a SpeedVac and suspended in 100 µl of 0.1 M triethylammonium bicarbonate, pH 8.5, before trypsination with 1 µg of trypsin gold at 37 °C on a shaker for about 16 h. Samples were purified and concentrated using homemade reversed phase tips.

MS-MS Data analyses of COMP peptides

For Q-TOF LC-MS (Q-TOF micro, Waters) mass spectrometric raw data were processed using Protein Lynx 2.1 (Waters). Peptide and neo-termini searches were performed using the databases (SwissProt 56.9 and ENSEMBL) and MASCOT MS/MS Ions Search (version 2.1). Due to the presence of collagens in tendon, hydroxylation of proline residues were allowed in database searches. MASCOT search parameters included: carbamidomethylation of cysteine as fixed modification, deamidation (Asn and Gln), and oxidation (Met and Pro) were considered as variable modifications. Other MASCOT

search parameters were: monoisotopic masses, ±0.2Da peptide mass tolerance, ±0.2Da fragment mass tolerance, max miss cleavage of 2, ion score minimum 20, only highest ranked peptide matches, and taxonomy *equus caballus*. MRM data were analyzed using the Skyline 1.4 software (MacCoss Lab Software, University of Washington).

Statistical analyses

Statistical analyses were conducted using SPSS PASW Statistics 18 (SPSS Inc Illinois, USA). Linear mixed models were used to analyse COMP release to account for effects of horse, experimental condition and time. Analyses for release and percentage change in release relative to the respective controls are shown for COMP. $p<0.05$ was considered statistically significant.

Ethics Statement

Ethical approval for the collection of post mortem equine tendons from an abattoir or local equine veterinary referral hospital for this study was sought and approved from the Ethics and Welfare Committee at the Royal Veterinary College (URN 2011 1117).

Results

Tendon explant viability *in vitro*

Confocal images illustrating explant viability are shown in Fig. 1. After culture for 24 h in serum deplete media, 90 ± 5% of cells within SDFT explants were viable. Explant viability was 60 ± 5% after 120 h in culture. The majority of cell death present was located along the periphery of the cut edges in a linear pattern along rows of tenocytes.

Effects of IL-1β and PGE_2 on the tendon ECM

Minimal collagen degradation by IL-1β and PGE_2 stimulation

Soluble collagens released from tendon explants treated with IL-1β or PGE_2 was minimal and ranged between 0.01-0.03 μg/mg of tissue in all samples between 24-96 h, but this was not significantly different from control cultures.

Release of COMP after IL-1β and PGE_2 stimulation

Mean COMP levels in media were 0.26 ± 0.1 μg/mg in the first 24 h equilibration period in unstimulated cultures. This 24 h period was not included in statistical analyses. The cumulative release of COMP significantly increased in all samples with time between 48-96 h ($p=0.008$) and was substantially increased by IL-1β (~10-fold increase) compared to control samples (Fig 2A). Although there was increased COMP release after stimulation with 1.0 μM PGE_2, this was not statistically significantly different compared to controls. COMP release was significantly reduced by addition of IL-1Ra (400 $ngml^{-1}$) and Firocoxib (1.0 μM) ($p<0.001$ and $p=0.004$ respectively) but not by Ilomastat (Fig 2B).

Analysis of ECM proteins in media by Western blotting

Analysis of culture medium from the tendon explant experiments confirmed the release of COMP from the tendon ECM over the 120 h culture period (Fig. 3). COMP was released in a number of known multimeric forms (25), which could be identified in non-reducing conditions (Fig. 3A-NR) and migrated as a single monomeric form in reducing conditions (Fig. 3A-R). The release of COMP from the tissue increased to a maximum at 48 h after explant cutting. Stimulation with 5 $ngml^{-1}$ IL-1β induced additional release of COMP as early as 15 h but was most marked after 48 h compared to controls (Fig. 3B R and NR) and included an approximately 100 kDa protein fragment not present in the control cultures under reduced conditions (Figs. 3 and 4). Fragments smaller than 100 kDa were observed with IL-1β after 15 h stimulation (Fig. 3). Qualitative assessment of western blots loaded with the same volume of media (Fig. 4) supported the increased release of both

monomeric (110 kDa) and fragmented COMP (~100 kDa) with 1.0 μM PGE_2, which was not significant by ELISA (Fig 2A). However fragments smaller than 100 kDa were only observed after stimulation at the higher PGE_2 dose (1.0 μM) and in contrast to IL-1β these fragments were present in relatively low abundance (Fig. 4). Combined addition of IL-1β with low or high doses of PGE_2 had no additional effect on COMP release.

Proteomic analyses of culture media by mass spectrometry

LC-MS and Q-TOF analyses

Semi quantitative LC-MS analyses showed COMP to be the most abundant ECM protein released in all media samples from stimulated and non-stimulated explants, followed by thrombospondin-4, clusterin and fibronectin (Table 1). Stimulation with IL-1β ($5ngml^{-1}$) induced a ~2-fold increase in COMP release compared to other experimental conditions. Consequently further analyses were focused on COMP and its related fragments. A list of neo-terminal peptides of COMP produced under different inflammatory stimuli is shown in Table 2. Five of the COMP neo-terminal peptides present in control samples were also common to samples stimulated with each pro-inflammatory mediator (i.e. present in all samples). Addition of IL-1β ($5ngml^{-1}$) generated a greater number of neo-terminal peptides of COMP compared to controls and stimulation with 1.0 μM PGE_2. The neo-terminal peptides generated from explants stimulated with 0.01 μM PGE_2 were identical to those released from controls. Interestingly, combined stimulation of tendon explants with IL-1β and low dose (0.01μM) PGE_2, generated a greater number of neo-terminal peptides in contrast to IL-1β with high dose (1.0μM) PGE_2 (Table 2).

Targeted mass spectrometry using MRM

MRM analyses were performed on experimental media samples after 72 h in culture from one experiment. Using this approach it was possible to perform relative quantifications of known peptides and neo-terminal fragments of COMP between samples stimulated with pro-inflammatory mediators and non-stimulated controls. There was a general trend for IL-1β stimulated tendon explants to release higher quantities of peptides compared to non-stimulated controls (Fig. 5A-F). The greatest numbers of peptides were generated when explants were incubated with both IL-1β and low dose (0.01 μM) PGE_2.

Proteomic analyses of normal and injured tendon

Analyses of normal and natural diseased flexor tendons identified differences in protein expression profiles for a large number of proteins as summarised in Fig. 6. Twenty one proteins were common to normal, sub-acute and chronic injured tendons, although a greater number of additional proteins were identified in injured samples, including albumin, Tenascin-C, fibronectin and Annexin's A1, A2, A5. COMP was identified in sub-acute and chronic injured tendons but was not the most abundant ECM protein in contrast to normal samples. COMP neo-terminal fragments detected in extracts of normal, sub-acute and chronic injured SDFTs are shown in Table 3. Four COMP neo-terminal fragments were identified in tissue extract samples of sub-acutely injured tendons and one in the chronic injury stage. Of these 5 COMP neo-terminal fragments identified in natural injury, C.AVGWAGNGLLCGR.D and F.CFSQENIIWANLR.Y were identified in the *in vitro* model system after stimulation with IL-1β. Quantitative MRM analyses showed that levels of the COMP neo-N-terminal fragment CFSQENIIWANLR, common to natural injury and the *in vitro* model were elevated in media from IL-1β stimulated tendon explants compared to non-stimulated controls and PGE_2 stimulated samples.

Discussion

Stimulation of tendon explants with two pro-inflammatory mediators did not induce significant collagen release between 24-96 h. Hence, tendon explants exhibit similar behaviour to cartilage with respect to the lack of collagens released during the early stages of culture (46,47). However it was not possible to maintain good cell viability in tendon explants beyond 5 days, which is why this time interval was selected as maximal for this study. The increased release of COMP with IL-1β supports a catabolic role of IL-1β in tendon similar to that reported for cartilage (28,39,40), which was further confirmed by the abrogating effects of IL-1Ra. Firocoxib but not Ilomastat inhibited IL-1β induced COMP release. Previous studies have shown that proteinases other than MMPs may be responsible for COMP degradation *in vitro* by aggrecanases such as ADAMTS-4 (28), which are not inhibited by Ilomastat, although the effects of aggrecanase inhibitors were not explored in the current study.

Although we did not investigate the effects of strain on tendon, stress deprivation has been shown to induce IL-1 production (48) and stress deprivation may occur in injury, which may explain some of the neo-terminal peptides observed in diseased tendon. COMP was readily released from the matrix, which may be either the consequence of weaker interactions with matrix components compared to other matrix proteins, or that the released COMP is newly synthesised. However, the presence of cleaved forms of COMP in control samples would be more consistent with a proteolytic mediated release. Whilst cell death may have released intracellular proteases, it is unlikely that this was the major source of fragments because our control samples differentiated those fragments generated or substantially elevated following cytokine addition. IL-1β stimulation enhanced the release of cleaved forms of COMP and further degradation of monomeric COMP (<100 kDa, Fig. 3B). Neither the low nor high dose of PGE_2 enhanced additional fragmentation patterns over control samples. However, MRM analyses showed trends for combined stimulation with IL-1β and PGE_2 that produced differing effects depending on the concentration of PGE_2. Stimulation with IL-1β and low dose PGE_2 resulted in increased release of cleaved peptides of ECM proteins, whilst IL-1β and high dose PGE_2 limited the number of neo-terminal cleavage sites. The synergy between low dose PGE_2 and IL-1β on peptide release in this study is curious and could be explained by a number of hypotheses. First, the kinetics of prostaglandin (EP) receptor occupancy may be prolonged by the higher concentration of PGE_2, leading to receptor desensitisation, which would dampen receptor effects. Second, the presence of higher levels of PGE_2 may exert an auto-regulatory feedback effect on IL-1 activity in order to modulate the inflammatory reaction (49). Third, the higher doses of PGE_2 can activate resolution of inflammation in tendon fibroblasts via the production of specialised pro-resolving mediators such as lipoxins (50). This has been reported in an identical experimental system whereby addition of 1.0 μM PGE_2 to normal tendon explants induced maximal LXA_4 release after 72 h in tissue culture (3). We have demonstrated combined stimulation of explants with IL-1β and the same concentrations of PGE_2 similarly induced LXA_4 release, with greater production with the higher dose of PGE_2, suggesting that PGE_2 may exert anti-catabolic effects on tendon ECM (36).

Comparative proteomic analysis of normal and naturally diseased tendons identified differences in protein / peptide profiles. The presence of Annexin A1 identified only in the sub-acute and chronic injury samples implicate both inflammatory and pro-apoptotic mechanisms are active (51), and continue into the later stages of tendon healing. COMP was also identified in samples of injured SDFT but it was not the most abundant protein, in contrast to normal tendons, suggesting a change in the tendon protein profile after

injury. Both injury phases had a similar number of proteins identified by proteomic analysis although the greater number of proteins unique to sub-acute disease suggests that a large change in the phenotype of tendon cells is a feature of early disease stages and this change to a large extent persists into chronic disease. This failure of the protein profile to return to normal suggests that injury permanently changes the composition of tendon ECM, which may compromise both the mechanical properties of the tissue and its influence on the responses of tenocytes in maintaining homeostasis. This may be a contributing factor to the high risk of re-injury in horses due to the formation of a repair scar with inferior mechanical properties to normal tendons (23,52).

MRM analyses showed that levels of the F.CFSQENIIWANLR.Y COMP peptide in sub-acute injury were also elevated after IL-1β stimulation of tendon explants. MRM analyses suggest that the relative abundance of the F.CFSQENIIWANLR.Y fragment is greater after stimulation with IL-1 β rather than PGE_2. Furthermore, the C.AVGWAGNGLLCGR.D COMP fragment was only identified after stimulation with IL-1β and not PGE_2 (low or high dose) and therefore the presence of these fragments *in vivo* provides supportive evidence that IL-1 is active in naturally occurring injury.

The identification of novel COMP peptide cleavage sites common to both natural disease and an *in vitro* model of tendon inflammation provide a platform for the development of antibodies to identify stage of tendon injury and enzyme inhibitors for therapeutic intervention. The combination of these disease specific fragments may allow a multiplex marker platform to be developed for tendon injury.

Competing Interests

The authors declare that no competing interests exist.

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Figure legends

Figure 1

Viability of tendon fibroblasts in explant culture. Confocal fluorescence micrographs of tendon sections treated with Calcein AM to denote cytoplasmic staining of live cells (green) and ethidium bromide for nuclear staining (red) of live and dead cells. Explants after (A) 24 h and (B) 120 h. Cell viability was 90 ± 5% after 24 h in culture and 60 ± 5% after 120 h. (C) Azide treated explant after 24h was used as a negative control. All experiments were performed within the 120 h culture period. Scale bar = 20μm.

Figure 2

Release of COMP into media from SDFT explants in culture. Normal SDFT explants were derived from 3 horses aged between 9-13 years (mean 10 ± 2 years). Explants were rested for 24 h after cutting prior to media replacement and stimulation commencing. Media were harvested at 48, 72 and 96 h with complete replacement at each time point. (A) Mean cumulative COMP release, showing significantly increased release with IL-1β stimulation alone and in combination with 20 μM Ilomastat. COMP release after stimulation with 1.0 μM PGE_2 was similar to non-stimulated controls. (B) Mean percentage change in COMP release relative to the respective control. Addition of either 400 $ngml^{-1}$ IL-1Ra or 1.0 μM Firocoxib significantly reduced IL-1β mediated COMP release. Error bars represent standard deviation. *p<0.05, **p<0.01, ***p<0.001.

Figure 3

COMP release into culture media from normal SDFT explants. Representative Western blots of media from experimental samples showing COMP release with time from control (A) and IL-1β stimulated tendon explants (B) under non-reduced (NR) and reduced (R) conditions. Densitometric analysis of protein bands is shown for non-reduced western blots. COMP release was enhanced by stimulation of explants with IL-1β compared to non-stimulated controls, with maximal release occurring at 48 h and the appearance of a number of distinct peptides <100 kDa. In IL-1β stimulated explants, monomeric COMP appears as a doublet under reduced conditions from 15-120 h after explant cutting, which is better demonstrated in a lower exposure of the blot in a lower panel. T= equine SDFT extract (total protein loaded 10μg) prepared in 4 M (GdnHCl) was used as a loading standard and as a positive control for COMP which is present mostly as a 550 kDa pentamer in the tissue.

Figure 4

Differential effects of IL-1β and PGE_2 on COMP release. Representative Western blot of media samples harvested at 4 h (A) and 48 h (B). Tendon extract = positive control (10 μg total protein); Control = media from unstimulated tendon at the same respective time point. COMP release and fragmentation were enhanced by stimulation with IL-1β at 48 h with lower molecular weight fragments present including the appearance of the 100 kDa peptide (doublet). Addition of PGE_2 resulted in the release of both intact and fragmented COMP (100 kDa) at 48 h. Smaller fragments were detected with the higher dose of PGE_2 but these were present in low abundance compared to stimulation with IL-1β.

Figure 5

MRM analyses of COMP peptides released into media after 72 h in culture: (A) AVAEPGIQLK, (B) ELQETNAALQDVR, (C) IDVCPENAEVTLTDFR, (D) LVPNPGQEDADR, (E) SSTGPGEQLR and (F) COMP neo-terminal fragment CFSQENIIWANLR. Each sample was run on the MRM in triplicate and the mean values with standard deviation are shown for each experimental condition. MRM analyses were conducted on triplicate samples from one experiment and not biological replicates, hence statistical analyses were not performed. Peak normalised area represents the summed area of the ion peak transitions for each peptide measured. There was a trend for enhanced COMP peptide/neo-N-terminal fragment release with IL-1β compared to non-stimulated controls. For all peptides, maximal release occurred after stimulation with both IL-1β and 0.01 μM PGE_2.

Figure 6

Venn representation of proteins identified from Q-TOF LC-MS. Samples of extracts of normal, sub-acute and chronic injured equine SDFT samples (n=3 for each) were analysed for global protein composition, demonstrating differences in tendon ECM protein expression profiles with injury stage. While a similar number of proteins were identified in both sub-acute and chronic injury phases, it was in the sub-acute phase injury that the greatest numbers of unique proteins were present.

Tables

Table 1

Top ranking identified tendon ECM proteins (by protein score) released into media by pro-inflammatory mediators. Protein abundance was measured by semi-quantitative LC-MS and is indicated by the exponentially modified protein abundance index in parenthesis (emPAI).

Rank of protein	**Control**	**IL-1β (5 ngml^{-1})**	**PGE_2 (0.01μM)**	**PGE_2 (1.0 μM)**
1	COMP (3.44)	COMP (6.41)	COMP (3.84)	COMP (3.08)
2	Thrombospondin 4 (1.35)	Thrombospondin 4 (1.7)	Thrombospondin 4 (1.28)	Thrombospondin 4 (0.92)
3	Fibronectin (0.6)	Clusterin (0.85)	Clusterin (0.85)	Clusterin (0.5)
4	Clusterin (0.5)	Fibronectin (0.51)	Fibronectin (0.74)	Fibronectin (0.49)
5	Decorin (0.09)	Interleukin-6 (0.56)	Collagen 3 (0.23)	Decorin (0.3)
6	Thrombospondin 1 (0.06)	Collagen 3 (0.17)	Thrombospondin 1 (0.06)	Collagen 3 (0.29)
7	CILP-1 (0.06)	Thrombospondin 1 (0.11)	CILP-1 (0.06)	Thrombospondin 1 (0.08)
8	Collagen 1 (0.06)	Aggrecan (0.05)	Aggrecan (0.03)	Aggrecan (0.02)

Table 2 Summary of Q-TOF LC-MS analyses for neo-terminal peptides of COMP released into media from stimulated tendon explants. Numbers indicate the position of the peptide within the equine COMP protein sequence (NCBI accession AF325902).

Control	**IL-1β**	**1.0 μM PGE_2**	**0.01 μM PGE_2 + IL-1β**	**1.0 μM PGE_2 + IL-1β**
228 - 238 C.PDGTPSPCHEK.A*	37 - 48 E.LQETNAALQDVR.E	89 - 108 Q.CAPGSCFPGVACTQTASGAR.C	37 - 48 E.LQETNAALQDVR.E	81 - 88 R.VSVRPLAQ.C
294 - 303 V.PNSGQEDADR.D*	88 - 108 A.QCAPGSCFPGVACTQTASGAR.C		88 - 108 A.QCAPGSCFPGVACTQTASGAR.C	89 - 108 Q.CAPGSCFPGVACTQTASGAR.C
642 - 649 S.TGPGEQLR.N *	89 - 108 Q.CAPGSCFPGVACTQTASGAR.C		89 - 108 Q.CAPGSCFPGVACTQTASGAR.C	
724 - 736 F.CFSQENIIWANLR.Y*	203 - 222 F.QCGPCQPGFVGDQASGCRPR.A		254 - 266 C.AVGWAGNGLLCGR.D	
726 – 736 F.SQENIIWANLR.Y *	254 - 266 C.AVGWAGNGLLCGR.D		725 – 736 C.FSQENIIWANLR.Y	
	269 - 279 T.DLDGFPDEKLR.C			
	320 - 330 V.PNEGDNCPLVR.N			
	600 - 613 F.GYQDSSSFYVVMWK.Q			

*Denotes the neo-terminal peptide was present in all samples, including 0.01 μM PGE_2 stimulated samples, which were the same as controls. Sequences shown with a dot represent the cleavage site and the residue following the dot represents the neo-terminus. IL-1β dose is 5 $ngml^{-1}$ for all samples.

Table 3
Summary of COMP peptides unique to natural SDFT injury. Numbers indicate the peptide position within the equine COMP protein sequence. [1]Peptides common to sub-acute tendon injuries and IL-1β stimulated tendon explants. [2]Levels of the F.CFSQENIIWANLR.Y peptide were increased in media from IL-1β stimulated tendon explants compared to controls.

Normal SDFT	Sub-acute SDFT injury	Chronic SDFT injury
228 - 238 C.PDGTPSPCHEK.A	254 - 266 C.AVGWAGNGLLCGR.D[1]	682 - 692 R.WFLQHRPQVGY.I
726 - 736 F.SQENIIWANLR.Y	652 - 663 A.LWHTGDTASQVR.L[1]	
	653 - 663 L.WHTGDTASQVR.L[1]	
	682 - 692 R.WFLQHRPQVGY.I	
	724 - 736 F.CFSQENIIWANLR.Y[2]	

Figures

Figure 1

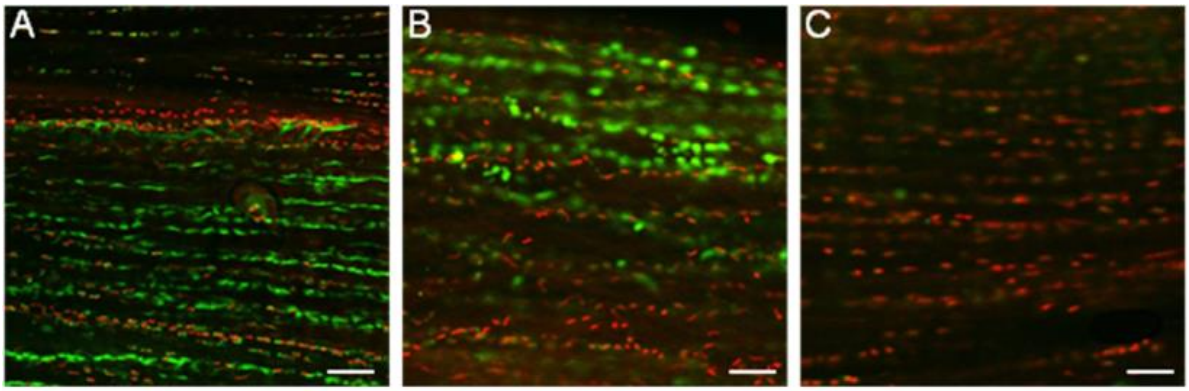

Figure 2

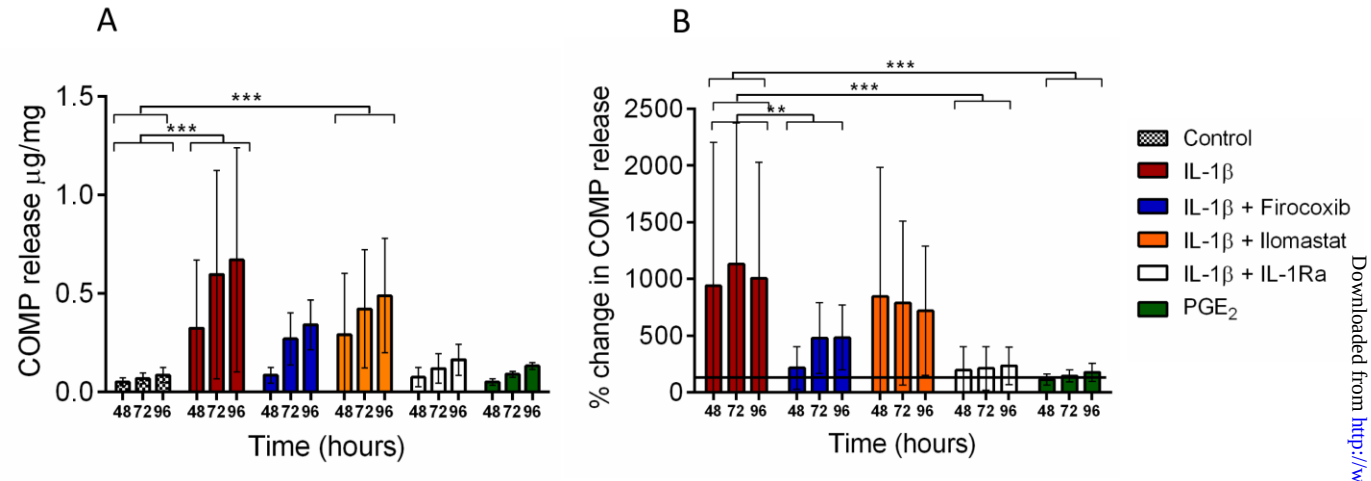

Figure 3

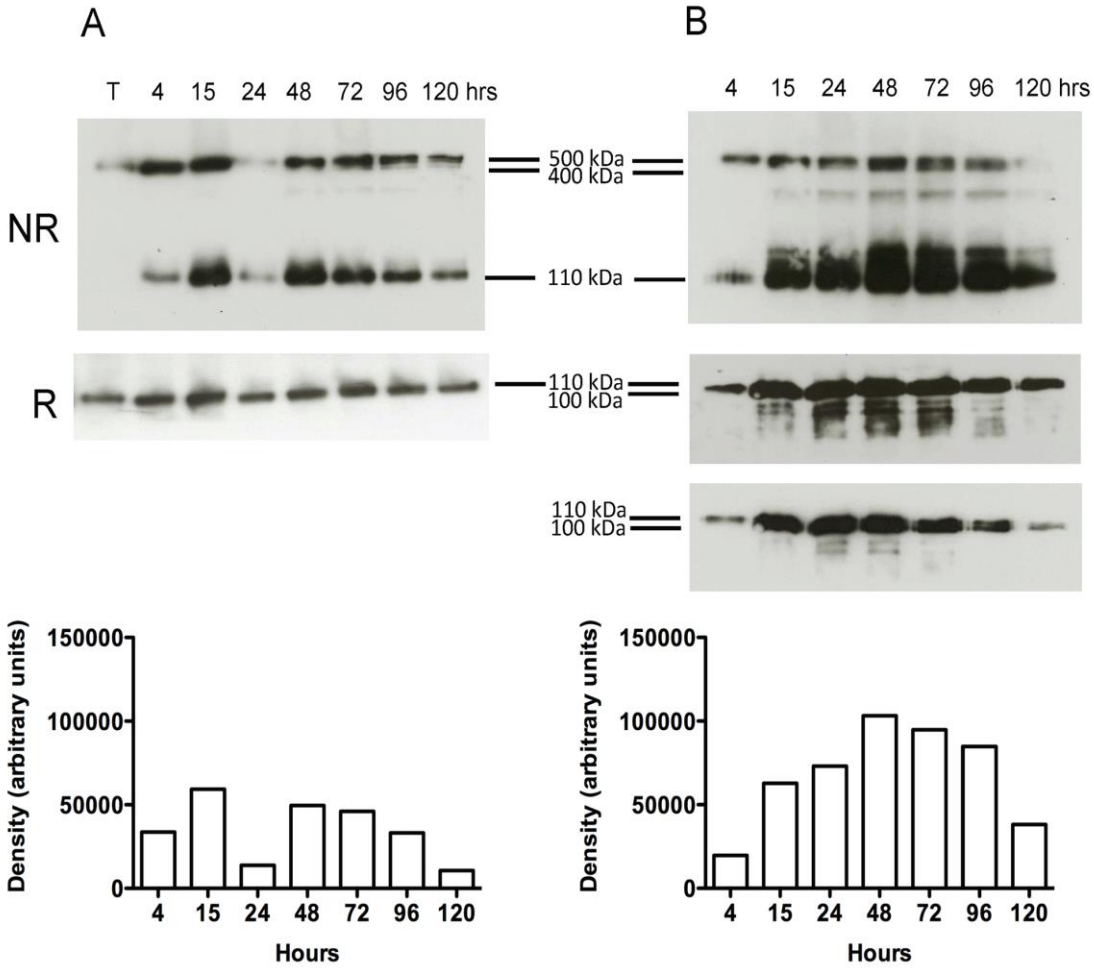

Figure 4

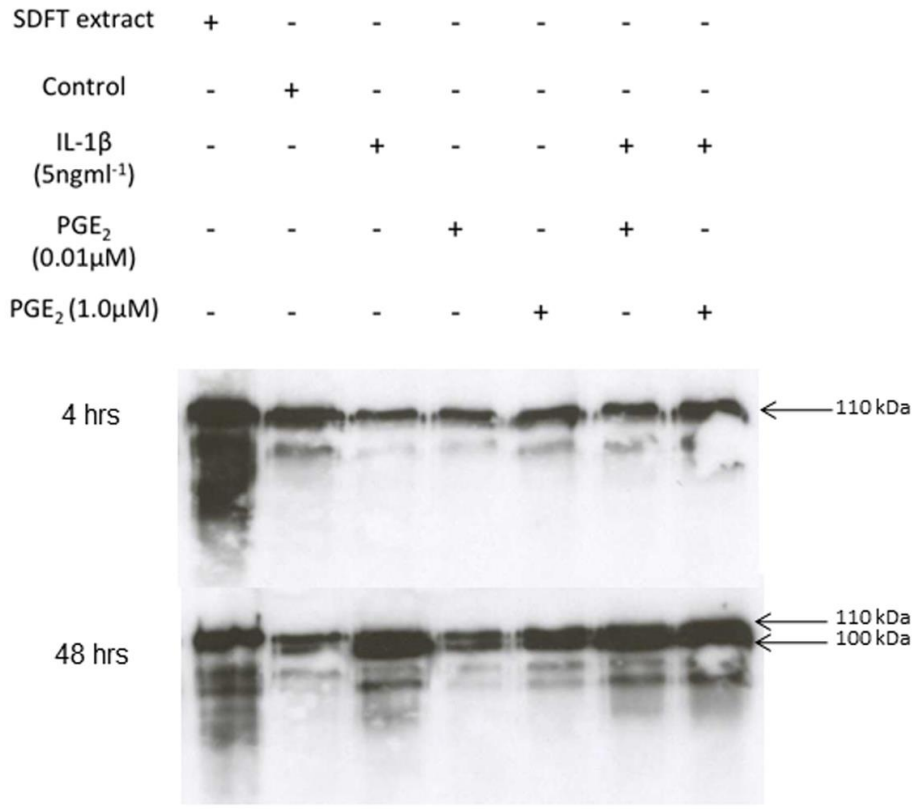

Figure 5

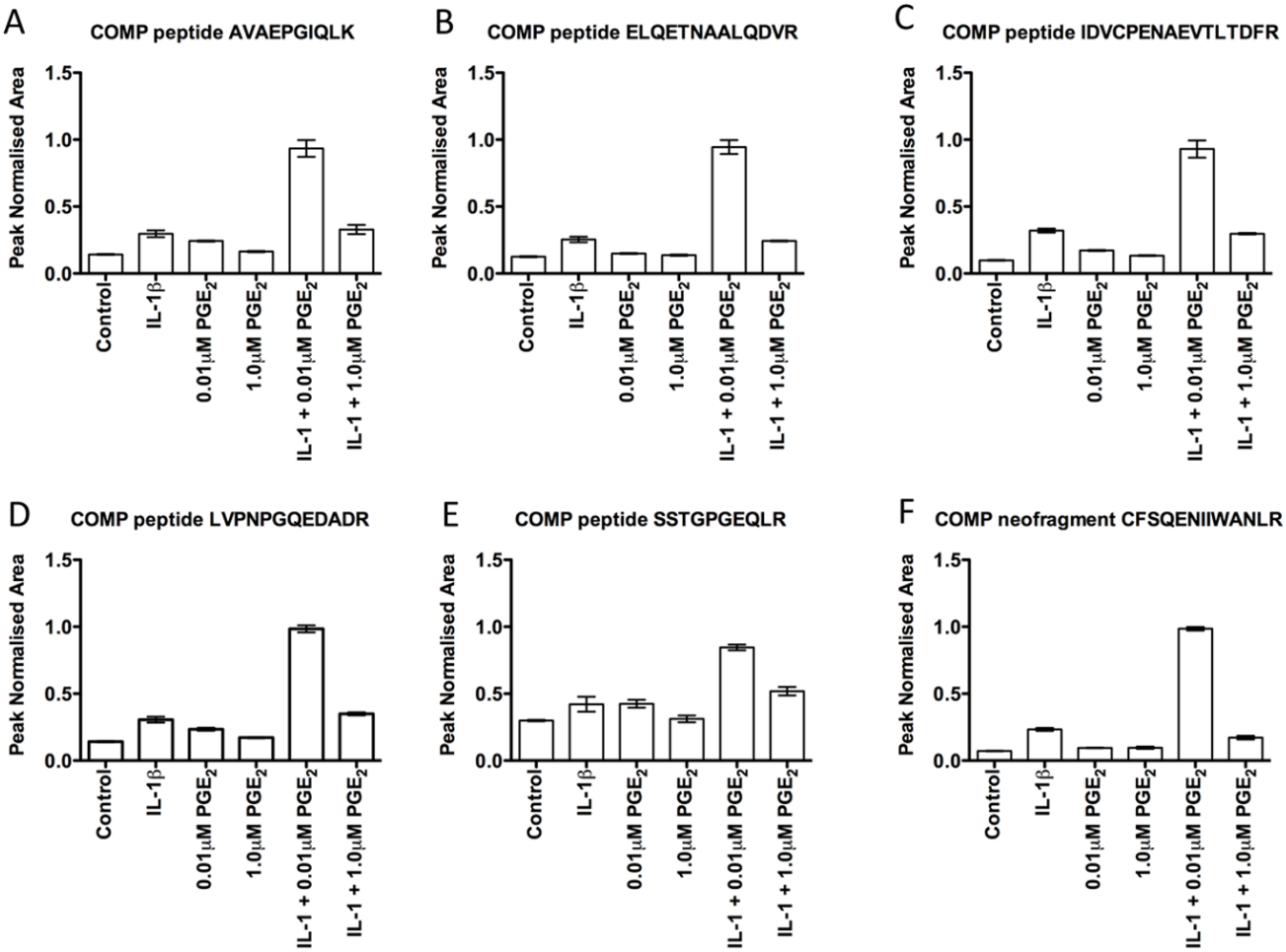

Figure 6