Proteomic Analysis of Tendon Extracellular Matrix Reveals Disease Stage-specific Fragmentation and Differential Cleavage of COMP (Cartilage Oligomeric Matrix Protein)*

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Background: Tendon disease is characterized by extensive remodeling 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.

During inflammatory processes the extracellular matrix (ECM) is extensively remodeled, 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 characterizing fragments released in naturally occurring disease, we hypothesized that stimulation of tendon explants with proinflammatory mediators in vitro would induce fragments of COMP analogous to natural disease. Therefore, normal tendon explants were stimulated with IL-1β and prostaglandin E2, and their effects on the release of COMP and its cleavage patterns were characterized. 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, whereas PGE2 had no catabolic effect. Of the cleavage fragments identified in early stage tendon disease, two 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.

Tendons have an abundant extracellular matrix (ECM)2 and are significant causes of morbidity in athletic individuals (1, 2).

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1 Author’s Choice—Final version full access.

2 The abbreviations used are: ECM, extracellular matrix; PG, prostaglandin; SDFT, superficial digital flexor tendon; Q-TOF, quadrupole TOF; MRM, multiple reaction monitoring; COMP, cartilage oligomeric matrix protein; MMP, matrix metalloproteinase.
COMP Fragmentation in Tendon Disease

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 intrathecral digital flexor tendon tears also show elevated COMP levels in tendon sheath fluids (31, 32). COMP degradation is mediated in part by matrix metalloproteinases (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 proinflammatory mediators such as IL-1 and PGE2 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). Whereas 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 to identify 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-1 and PGE2 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-bred horses aged between 2 and 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 before euthanasia of the horse. Tendons were classified as normal based on their macroscopic postmortem appearance, which included lack of visible signs of swelling of the tendon body and a consistent pattern of fascicles on hematoxylin- 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 and 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 three parallel sterile microtome blades (Surgipath) inserted into a custom-designed cutting template to create 2 × 2 × 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) in tissue culture 6-well plates (VWR) at 37 °C in humidified atmosphere (5% CO2 and air). This method of preparing the explant tissue consistently produced average wet weights of 300 mg (±30 mg).

To assess the effects of proinflammatory mediators on release of tendon matrix components, explants were stimulated with human recombinant IL-1β (5 ng/mL) (Calbiochem) or PGE2 (0.01 or 1.0 μM) (Sigma), and release of total collagen and COMP into tissue culture media was quantified and compared with non-stimulated controls. After cutting (time 0), explants were incubated in serum-depleted 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 were stimulated with proinflammatory mediators. The following inhibitors were added to the experimental system to identify inflammation-relevant release of COMP by intervention of the PGE2 synthesis pathways (including PGE2 synthesis via IL-1β): 1.0 μM Firocoxib (Merial, France), 20 μM Ilomastat (Calbiochem), 400 ng/mL recombinant equine IL-1Ra (R&D Systems). Media were harvested and analyzed 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′,6-diamidino-2-phenylindole (DAPI) and calcein AM (Sigma) in PBS containing 5.6 mM glucose, 0.5 mM MgCl2, and 0.9 mM CaCl2 for 1 h in dark conditions before confocal microscopy (Leica Microsystems, Milton Keynes, UK). Viability of explants cultured in serum-depleted DMEM containing 5% penicillin and streptomycin were compared at 24 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) 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 microtiter plates, and the final absorbance was 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 was 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 proinflammatory 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 the addition of dithiothreitol to 0.1 M and heated to 95 °C for 5 min before electrophoresis on 8–10% SDS/PAGE gels. After electrophoresis, proteins were transferred for Western blotting (Bio-Rad) onto PVDF membranes (GE Healthcare). 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). After washing in TBST (3 times for 10 min 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 3 times as before and incubated with anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology) in antibody buffer for 2 h at a 1:2000 dilution. Antibody-positive protein bands were visualized 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 artifacts.

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 an 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 was stimulated with proinflammatory mediators for a further 24 h. All media samples for proteomic analyses were analyzed at the 72 h time point after explant cutting.

Preparation of Media Samples for the Q-TOF MS—Media samples (10 μl of trypsin digest, see above) were cleaned with reversed-phase C18 columns according to the manufacturer’s instructions (SUM SS18V); columns were purchased from the Nest Group.

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) were homogenized in a buffer containing protease inhibitors (1:100 dilution of protease inhibitor mixture III, Calbiochem) and 10 mM EDTA. Samples were then centrifuged at 56 °C on a shaker for 15 min at 13,000 g and subsequently dialyzed in 20 μM formic acid before injection onto the Q-TOF MS.

Preparation of Media Samples for the Triple Quadrupole MS—Media samples (10 μl of trypsin digest, see above) were cleaned with reversed-phase C18 columns according to the manufacturer’s instructions (SUM SS18V); columns were purchased from the Nest Group.

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 dithiothreitol and agitated at 56 °C for 30 min. Samples were alkylated with 16 mM iodoacetamide at room temperature in the dark for 1 h. Samples were digested with 0.5 μg trypsin gold (Promega, Madison, WI) overnight at 37 °C on a shaker for 16 h. Samples were then centrifuged in a SpeedVac and suspended in 100 μl of 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 dialyzed in 20 μl 0.2% formic acid before injection onto the Q-TOF MS.

Preparation of Media Samples for the Triple Quadrupole MS—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 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 proinflammatory 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 the addition of dithiothreitol to 0.1 M and heated to 95 °C for 5 min before electrophoresis on 8–10% SDS/PAGE gels. After electrophoresis, proteins were transferred for Western blotting (Bio-Rad) onto PVDF membranes (GE Healthcare). 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). After washing in TBST (3 times for 10 min 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 3 times as before and incubated with anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technology) in antibody buffer for 2 h at a 1:2000 dilution. Antibody-positive protein bands were visualized 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 artifacts.

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Preparation of Media Samples for the Triple Quadrupole MS—Media samples (10 μl of trypsin digest, see above) were cleaned with reversed-phase C18 columns according to the manufacturer’s instructions (SUM SS18V); columns were purchased from the Nest Group.
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, ±0.2-Da peptide mass tolerance, ±0.2-Da 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.). Linear mixed models were used to analyze 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 PGE2 on the Tendon ECM

Minimal Collagen Degradation by IL-1β and PGE2, Stimulation—Soluble collagens released from tendon explants treated with IL-1β or PGE2 was minimal and ranged between 0.01 and 0.03 µg/mg of tissue in all samples between 24 and 96 h, but this was not significantly different from control cultures.

Release of COMP after IL-1β and PGE2 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 times between 48 and 96 h (p = 0.008) and was substantially increased by IL-1β (~10-fold increase) compared with control samples (Fig. 2A). Although there was increased COMP release after stimulation with 1.0 µM PGE2, this was not statistically significantly different compared with controls. COMP release was significantly reduced by the addition of IL-1Ra (400 ng/ml) 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) that 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 explan cutting. Stimulation with 5 ng/ml IL-1β induced additional release of COMP as early as 15 h but was most marked after 48 h compared with controls (Fig. 3B, R and NR) and included an ~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 of 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 PGE2, which was not significant by ELISA (Fig. 2A). However, fragments smaller than 100 kDa were only observed after stimulation at the higher PGE2 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 PGE2 had no additional effect on COMP release.

Proteomic Analyses of Culture Media by Mass Spectrometry

LC-MS and Q-TOF Analyses—Semiquantitative 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 (5 ng/ml) induced an ~2-fold increase in COMP release compared with 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 detected with the higher dose of PGE2, but these were present in low abundance compared with stimulation with IL-1β.

TABLE 1

| Rank of protein | Control | IL-1β (5 ng/ml) | PGE2 (0.01 µM) | PGE2 (1.0 µM) |
|-----------------|---------|----------------|----------------|---------------|
| 1               | COMP (3.44) | COMP (6.41) | COMP (3.84) | COMP (3.08) |
| 2               | Thrombospondin4 (1.35) | Thrombospondin4 (1.7) | Thrombospondin4 (1.28) | Thrombospondin4 (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) | Collagen3 (0.23) | Decorin (0.2) |
| 6               | Thrombospondin1 (0.06) | Collagen3 (0.17) | Thrombospondin1 (0.06) | Collagen3 (0.29) |
| 7               | CILP-1 (0.06) | Thrombospondin1 (0.11) | CILP-1 (0.06) | Thrombospondin1 (0.08) |
| 8               | Collagen1 (0.06) | Aggrecan (0.05) | Aggrecan (0.03) | Aggrecan (0.02) |

Figure 4. Differential effects of IL-1β and PGE2 on COMP release. Representative Western blot of media samples harvested at 4 h and 48 h. 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). The addition of PGE2 resulted in the release of both intact and fragmented COMP (100 kDa) at 48 h. Smaller fragments were detected with the higher dose of PGE2, but these were present in low abundance compared with stimulation with IL-1β.

FIGURE 4. Differential effects of IL-1β and PGE2 on COMP release. Representative Western blot of media samples harvested at 4 h and 48 h. 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). The addition of PGE2 resulted in the release of both intact and fragmented COMP (100 kDa) at 48 h. Smaller fragments were detected with the higher dose of PGE2, but these were present in low abundance compared with stimulation with IL-1β.

TABLE 1

Top ranking identified tendon ECM proteins (by protein score) released into media by proinflammatory mediators

Protein abundance was measured by semi-quantitative LC-MS and is indicated by the exponentially modified protein abundance index in parentheses (emPAI).
### Table 2

Summary of Q-TOF LC-MS analyses for neo-terminal peptides of COMP released into media from stimulated tendon explants

| Control | IL-1β | 0.01 μM PGE₂ + IL-1β | 1.0 μM PGE₂ + IL-1β |
|---------|-------|----------------------|---------------------|
| 228–238 C ↓ PDGTPSCEHK ↓ A² | 37–48 E ↓ LQETNAAQDVR ↓ E | 89–108 Q ↓ CAPGSCFPG VACTQTASGR ↓ C | 81–88 R ↓ VSVRPLAQ ↓ C |
| 294–303 V ↓ PNSGQEDADR ↓ D² | 88–108 A ↓ QCAPGSCFPGVACTQTASGR ↓ C | 88–108 A ↓ QCAPGSCFPGVACTQTASGR ↓ C | 89–108 Q ↓ CAPGSCFPGV ACTQTASGR ↓ C |
| 642–649 S ↓ TGPEQWR ↓ N² | 89–108 Q ↓ CAPGSCFPGVACTQTASGR ↓ C | 89–108 Q ↓ CAPGSCFPGVACTQTASGR ↓ C | 254–266 C ↓ AVGWAGNGLLGR ↓ D |
| 724–736 F ↓ CSQENIWNALR ↓ Y² | 203–222 F ↓ QQPCQPGQVDGQDASGPRR ↓ A | 254–266 C ↓ AVGWAGNGLLGR ↓ D | 269–279 T ↓ DLDGPDPDEKR ↓ Q |
| 726–736 F ↓ SQENIWNALR ↓ Y² | 254–266 C ↓ AVGWAGNGLLGR ↓ D | 254–266 C ↓ AVGWAGNGLLGR ↓ D | 725–736 C ↓ FSVQENIWNALR ↓ Y |
| 600–613 F ↓ GYQDSSSFYVVMW ↓ Q | 320–330 V ↓ PNEDGNCPLVR ↓ N | 600–613 F ↓ GYQDSSSFYVVMW ↓ Q | |

² Denotes the neo-terminal peptide was present in all samples, including 0.01 μM PGE₂-stimulated samples, which were the same as controls. Sequences shown with an arrow represent the cleavage site, and the residue following the arrow represents the neo-terminus. IL-1β dose is 5 ng/ml. PGE₂ dose is 5 ng/ml.

### Discussion

Simulation of tendon explants with two pro-inflammatory mediators did not induce significant collagen release between 24 and 96 h. Hence, tendon explants exhibit similar behavior to the early stages of healing in vivo. The increase in tendon explants compared to IL-1β-stimulated controls, which is why this time interval was selected as maximal for this study. The increased release of COMP with IL-1β supports a carboxyl role of IL-1β, which was further confirmed by the analysis of tendon explants with high dose (1.0 μM) PGE₂-stimulated samples.

### Proteomic Analyses of Normal and Injured Tendon

#### COMP Fragmentation in Tendon Disease

Proteomic analyses of normal and injured flexor tendons identified differences in protein expression profiles for large numbers of proteins as summarized in Fig. 6. Forty-two proteins were common to normal and injured explants, although a greater number of additional proteins were identified in injured samples, including annexin A1, Annexin A2, and Annexin A5. COMP was identified in tissue extracts of normal, sub-acute, and chronic injured SDFTs, and it was not found in the acute stage. Of these five COMP neo-terminal fragments identified in tissue extracts of normal, sub-acute, and chronic injured SDFTs, common to natural injury, CFSQENIWNALR, was identified in injured samples of sub-acutely injured tendons and one in the chronic injury stage. Of these five COMP neo-terminal fragments identified in tissue extracts of normal, sub-acute, and chronic injured SDFTs, common to natural injury, CFSQENIWNALR, was identified in sub-acute and chronic injured SDFTs, and COMP was identified in tissue extracts of normal, sub-acute, and chronic injured SDFTs. There was a general trend for IL-1β-mediated collagen release to be higher in high dose (1.0 μM) PGE₂-stimulated samples compared to low dose (0.01 μM) PGE₂-stimulated controls.

#### Targeted Mass Spectrometry Using MRM

Targeted mass spectrometry using MRM revealed higher quantities of peptides identified in samples from explants stimulated with 0.01 μM PGE₂ (Fig. 4). The greatest number of peptides were identified in samples from explants stimulated with 1.0 μM PGE₂. The neo-terminal peptides were identified from explants stimulated with 0.01 μM PGE₂, and low dose (0.01 μM) PGE₂ generated a greater number of neo-terminal peptides compared to controls. The addition of IL-1β generated a greater number of neo-terminal peptides common to samples stimulated with each pro-inflammatory mediator (i.e. present in all samples). The addition of IL-1β generated a greater number of neo-terminal peptides compared to controls. The addition of IL-1β generated a greater number of neo-terminal peptides compared to controls.
IL-1β-induced COMP release. Previous studies have shown that proteinases other than matrix metalloproteinases 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 with other matrix proteins or that the released COMP is newly synthesized. However, the presence of cleaved forms of COMP in control samples would be more consistent with a proteolytic-mediated release. Although 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 after 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 PGE2 enhanced additional fragmentation patterns over control samples. However, MRM analyses showed trends for combined stimulation with IL-1β and PGE2 that produced differing effects depending on the concentration of PGE2. Stimulation with IL-1β and low dose PGE2 resulted in increased release of cleaved peptides of ECM proteins, whereas IL-1β and high dose PGE2 limited the number of neo-terminal cleavage sites. The synergy between low dose PGE2 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 receptor occupancy may be prolonged by the higher concentration of PGE2, leading to receptor desensitization, 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 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 specialized pro-resolving mediators such as lipoxins (50). This has been reported in an identical experimental system whereby the addition of 1.0 µM PGE$_2$ to normal tendon explants induced maximal lipoxin A$_4$ 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 lipoxin A$_4$ release, with greater production with the higher dose of PGE$_2$, suggesting that PGE$_2$ can activate 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 implicates that 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 PGE$_2$ may exert its effects on tendon ECM (36).

MRM analyses showed that levels of the F↓CFSEQNIWANLR↓Y 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↓CFSEQNIWIWANLR↓Y fragment is greater after stimulation with IL-1β rather than PGE$_2$. Furthermore, the C↓AVGWAGNLLGCR↓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 tendon injury.

The identification of novel COMP peptide cleavage sites common to both natural disease and in vitro model of tendon inflammation provide a platform for the development of antibodies to identify the 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.

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### TABLE 3

**Summary of COMP peptides unique to natural SDFT injury**

| Normal SDFT | Sub-acute SDFT injury | Chronic SDFT injury |
|-------------|-----------------------|---------------------|
| 228–238 C ↓ PDGTSPSCHEK ↓ A | 254–266 C ↓ AVGWAGNLLGCR ↓ D$^b$ | 682–692 R ↓ WFLQHRPQGY ↓ L$^a$ |
| 726–736 F ↓ SQENIIWANLR ↓ Y | 652–663 A ↓ LWHGDATASQVR ↓ L$^b$ | 682–692 R ↓ WFLQHRPQGY ↓ I |
| 653–661 L ↓ WHTGDATASQVR ↓ L$^a$ | 724–736 F ↓ CFSEQNIWANLR ↓ Y$^a$ |

$^a$ Peptides common to sub-acute tendon injuries and IL-1β-stimulated tendon explants.

$^b$ Levels of the F ↓ CFSEQNIWANLR ↓ Y peptide were increased in media from IL-1β stimulated tendon explants compared to controls.
