Synovium secretome as a disease-modifying treatment for equine osteoarthritis

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OBJECTIVE
To identify chondroprotective factors as potential disease-modifying osteoarthritis treatments using an unbiased, bottom-up proteomics approach.

SAMPLES
Paired equine cartilage explants and synovial membrane were collected postmortem from 4 horses with no history of lameness and grossly normal joints at necropsy.

PROCEDURES
Six groups were established: cartilage, synoviocytes, and cartilage + synoviocytes (coculture), all with or without interleukin (IL)-1β. The catabolic effect of IL-1β was verified by glycosaminoglycan (GAG) released from cartilage into media by 1,9-dimethyl-methylene blue assay and cartilage toluidine blue histochemistry. Conditioned media from cocultures with or with IL-1β were submitted for bottom-up proteomic analysis. Synoviocyte gene expression was evaluated using reverse transcription–quantitative PCR (RT-qPCR) for proteins of interest identified in the proteomics scan.

RESULTS
GAG content was retained in cartilage when in cocultures treated with IL-1β. Fourteen proteins of interest were selected from the proteomic analysis. From these 14 proteins, metalloproteinase inhibitor 3 precursor (TIMP3), tumor necrosis factor receptor superfamily member 11B (TNFRSF11B), insulin-like growth factor-binding protein 2 (IGFBP2), and alpha-2 macroglobulin (A2M) were selected for synoviocyte gene expression analysis by RT-qPCR. Gene expression of TIMP3 (P = .02) and TNFRSF11B (P = .04) were significantly increased in synoviocytes from cocultures treated with IL-1β compared to controls. Contrary to expectations based on protein expression, IGFBP2 gene expression (P = .04) was significantly decreased in IL-1β-stimulated coculture synoviocytes compared to control coculture synoviocytes. A2M gene expression in synoviocytes was not different between coculture groups.

CLINICAL RELEVANCE
The secretome from synoviocytes could provide a milieu of bioactive factors to restore joint homeostasis in osteoarthritis.
contains 1 or more bioactive mediators that protect cartilage from the degradative effects of IL-1β. However, these synovium secretome factors have not been identified. In this study, we aimed to identify synovium secretome factors using an unbiased, bottom-up proteomics approach. Identifying 1 or more unique molecules, or the entire secretome, could then be explored further as disease-modifying OA drugs for human and veterinary patients.

Materials and Methods

Study design
Tissue samples were collected from horses that were euthanized using IV pentobarbital (~1 mL/4.5 kg) as part of other projects at the institution. Samples were collected postmortem, with Institutional Animal Care and Use Committee approval. No animal was receiving medication for at least 2 months prior to euthanasia. Broadly, cocultures of cartilage and synoviocytes were treated with and without IL-1β. Conditioned media containing the synoviocyte secretome were subjected to proteomic analysis. Relative protein abundance was calculated for IL-1β-treated cocultures versus control cocultures. Gene expression for proteins of interest was verified in synoviocytes by reverse transcription–quantitative PCR (RT-qPCR).

Cartilage and synoviocyte cocultures
Cartilage and synovial membrane were harvested from the patellofemoral joints of 4 horses euthanized for reasons unrelated to joint disease or this study (age range, 7 months to 7 years). All joints were visually normal. Paired full-thickness cartilage explants and synoviocytes were isolated to establish cocultures with synoviocytes on the bottom of the well, and cartilage explants were suspended in the transwell as described previously. Cultures were established in Dulbecco’s Modified Eagle Medium with 10% fetal bovine serum, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 50 μg/mL ascorbic acid, 300 μg/mL L-glutamine, 100 U/mL penicillin sodium, and 100 μg/mL streptomycin sulfate at 37°C with 5% carbon dioxide and 90% humidity. After 24 hours, media were replaced with serum-free medium ± recombinant equine IL-1β (10 ng/mL, R&D Systems). Media were again exchanged after 48 and 96 hours, and the resultant conditioned medium was collected. Protease inhibitors were added (Protease Inhibitor Cocktail tablets; Roche Holding AG), and samples were centrifuged at 350 X g for 3 minutes to remove particulate matter and stored at -80°C until proteomic and GAG analyses were performed. Cartilage explants were fixed in 10% neutral buffered formalin for toluidine blue histochemistry; synoviocytes were lysed for gene expression analyses.

Toluidine blue histochemistry
Three cartilage explants per culture well were fixed in 10% neutral buffered formalin for 24 hours, processed, embedded in paraffin, sectioned (5 μm), and stained with toluidine blue to evaluate glycosaminoglycan distribution.

Bottom-up proteomics
The aim of our study was to identify proteins secreted by synoviocytes that protect cartilage from the catabolic effects of IL-1β. Therefore, the proteomic analysis was designed a priori to identify proteins that were increased in IL-1β-treated cocultures relative to control cocultures. Absolute protein quantification was not performed. Proteins were identified using Orbitrap Fusion by FT-Q-IT mode for solution-based protein identifications and associated label-free quantitation (LFQ).

In-solution digestion was performed on an S-Trap microcartridge column (ProtiFi) according to protocol, as described previously, with slight modification. Twenty micrograms of the protein samples in 25 μL buffer consisting of 50 mM tetraethylammonium bromide (TEAB, pH 8.5), 6 M urea, 2 M thiourea, and 1% SDS were reduced with 10 mM Tris(2-carboxyethyl) phosphine hydrochloride for 1 hour at 34°C, then alkylated with 50 mM iodoacetamide for 1 hour in the dark and quenched with a final concentration of 36 mM dithiothreitol. After quenching, 12% phosphoric acid was added to a final concentration of 1.2%, followed by a 1:7 dilution (volume/volume) with binding buffer (90% methanol, 0.1 M TEAB, pH 8.5). The samples were then loaded into the S-Trap column cartridges and centrifuged at 4,000 X g for 30 seconds, and washed three times with 150 μL binding buffer by centrifuge at 4,000 X g for 30 seconds. Digestion was performed with 2 μg trypsin (1:10 weight/weight) in 125 μL 50 mM TEAB (pH 8.5) added to the top of the spin column and incubated at 37°C overnight. After incubation, the digested peptides were eluted off the S-Trap column sequentially, with 80 μL each of 50 mM TEAB (pH 8.5) followed by 0.2% formic acid (FA) and 50% acetonitrile in 0.2% FA. Three eluted peptides washes were pooled together and evaporated to dryness using a Speedvac SC110 (Thermo Savant).

Protein identification was performed by nanoscale liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS) analysis. The tryptic digests were reconstituted in 85 μL of 0.5% FA for nanoLC-MS/MS analysis, which was carried out using an Orbitrap Fusion Tribrid mass spectrometer (Thermo-Fisher Scientific) equipped with a nanospray Flex Ion Source and coupled with a Dionex UltiMate 3000 RSLc nano system (Thermo-Fisher Scientific). The peptides (4 μL) were injected onto a PepMap™ C-18 Reverse Phase (C-18 RP) nano-trapping column (5 μm, 100 μm internal diameter X 20 mm) at a 20-μL/minute flow rate for rapid sample loading, and then separated on a PepMap™ C-18 RP nanocolumn (2 μm, 75 μm X 25 cm) at 35°C. The peptides were eluted in a 90-minute gradient of 7% to 38% acetonitrile (ACN) in 0.1% FA at 300 nL/minute, followed by an 8-minute ramping to 90% ACN–0.1% FA and an 8-minute hold at 90% ACN–0.1% FA. The column was re-equilibrated with 0.1% FA for 25 minutes prior to the next injection. The Orbitrap Fusion
was operated in positive ion mode with the spray voltage set at 1.6 kV and the source temperature set at 275°C. External calibration for FT, IT, and quadrupole mass analyzers was performed. For the data-dependent acquisition analysis, the instrument was operated using an FT mass analyzer and MS scan to select precursor ions, followed by 3-second “Top Speed” data-dependent collision-induced dissociation ion trap MS/MS scans at 1.6 m/z quadrupole isolation for precursor peptides with multiple charged ions above a threshold ion count of 10,000 and a normalized collision energy of 30%. MS survey scans were set at a resolving power of 120,000 50% of the maximum peak height (full width at half maximum) at m/z 200, for the mass range of m/z 375 to 1,575. Dynamic exclusion parameters were set at 50 seconds of exclusion with ± 10 ppm exclusion mass width. All data were acquired using Xcalibur 3.0 operation software (Thermo Fisher Scientific).

**Data analysis**

The data-dependent acquisition raw files for collision-induced dissociation MS/MS were subjected to database searches using Proteome Discoverer (PD) 2.3 software (Thermo Fisher Scientific) with the Sequest HT algorithm. The PD 2.3 processing workflow containing an additional node of the Minora Feature Detector for precursor ion-based quantification was used for protein identification and protein relative quantitation analysis between samples. The database search was conducted against an Equus caballus database containing 245,782 sequences downloaded from the National Center for Biotechnology Information. Two missed trypsin cleavage sites were allowed. The peptide precursor tolerance was set to 10 ppm; fragment ion tolerance was set to 0.6 Da. Variable modifications of methionine oxidation, deamidation of asparagine/glutamine, N-terminal acetylation, and fixed modification of cysteine carbamidomethylation were set for the database search. Only high-confidence peptides defined by Sequest HT with a 1% of false discovery rate by Percolator were considered for the peptide identification. The final protein identifications contained protein groups that were filtered with at least 2 peptides per protein. Relative quantitation of identified proteins between the IL-1β-treated and control conditioned media samples was determined by the LFQ workflow in PD 2.3. The precursor abundance intensity for each peptide identified by MS/MS in each sample was determined automatically, and the unique plus razor peptides for each protein in each sample were summed and used for calculating the protein abundance by PD 2.3 software. Protein ratios were calculated based on a pairwise ratio for treated versus control samples.

**Search Tool for the Retrieval of Interacting Genes/Proteins**

Uniprot accession numbers of proteins of interest were subjected to database searches using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) for visualization of the network between proteins, with an abundance ratio > 1 in coculture media with or without IL-1β. STRING (version 11.5, August 12, 2021; ELIXIR’s Core Data Resources) uses biologic databases from numerous sources, and Web resources of known and predicted protein–protein interactions.

**Synoviocyte gene expression**

Synoviocytes were lysed, and total RNA was isolated using the RNeasy Mini Kit (QIAGEN). Purity and concentration of the RNA was assessed by UV microspectrophotometry (NanoDrop 1000 Spectrophotometer; Thermo Scientific). Based on proteomic results, gene expression of insulin-like growth factor-binding protein 2 (IGFBP2), metalloproteinase inhibitor 3 precursor (TIMP3), and tumor necrosis factor receptor superfamily member 11B (TNFRSF11B) were quantified by RT-qPCR using equine-specific primers and probes (Supplemental Table 1) and the Taqman One-Step RT-qPCR technique (ViiA 7 Real-Time PCR System, Applied Biosystems). The copy number of messenger RNA was determined using absolute qPCR derived from a standard curve developed for each gene at the time of analysis, and these values were normalized to the reference gene, 18S.

**Statistical analysis**

To compare gene expression in synoviocytes between IL-1β-treated and control cocultures, a paired sample t test was performed. A P value < .05 was considered significant.

**Results**

**Toluidine blue histochemistry**

To verify that synoviocytes protected cartilage from the catabolic effects of IL-1β, matrix metachromasia as an indicator of GAG content was examined in cartilage explants. In cartilage-only control cultures, matrix GAG was intense and evenly distributed throughout the explant (Figure 1). As anticipated, addition of IL-1β resulted in marked GAG loss, with complete GAG depletion in the superficial two thirds of the explants and with minimal GAG remaining in the deeper one third of the explants. In cartilage cocultured with synoviocytes, matrix metachromasia was similar or increased compared to cartilage-only cultures, and when cocultures were treated with IL-1β, GAG staining was similar to cartilage-only controls in the deeper layers, with GAG loss confined to the superficial one third of the explant. These results confirmed that the presence of synoviocytes protected cartilage from IL-1β-induced matrix GAG loss. Spectrophotometric quantification of GAG loss was not performed because the goal of our study was to perform proteomic analysis. Histologic confirmation of IL-1β-induced cartilage matrix GAG loss confirmed that the culture conditions were appropriate to continue with proteomic analysis.

**Proteomic analysis of conditioned media**

The aim of our study was to identify proteins secreted from synoviocytes that protected cartilage...
from IL-1β-induced catabolism, so only conditioned media from cocultures were used for proteomics. In conditioned media, 639 proteins met the criteria of at least 2 unique peptides with 95% confidence. To identify proteins that were upregulated in IL-1β-stimulated cocultures, an abundance ratio was calculated as protein “X” in cocultures with IL-1β versus protein “X” in cocultures without IL-1β. Unique proteins (n = 132) with an abundance ratio > 1 were identified (Supplemental Table 2) and classified by their molecular function as extracellular matrix, 51%; structural, 18%; catabolic, 15%; extracellular matrix/structural, 12%; chemokine, 2%; or growth factor, 2%. Structural proteins such as keratin, collagen, and actin were deemed of less interest, iterating the list to 14 proteins of interest (Table 1). Functional protein–protein association networks were assessed using STRING analysis (Figure 2). From these 14 proteins, based on a subjective combination of abundance ratios, known role in cartilage biology, and the STRING analysis, TIMP3, IGFBP2, TNFRSF11B (aka osteoprotegerin [OPG]), and alpha-2 macroglobulin (A2M) genes were selected to be measured in synoviocytes by qPCR.

**Synoviocyte gene expression**

TIMP3 is a potent inhibitor of matrix-degrading aggrecanases and collagenases. Synoviocyte TIMP3 expression (P = .02) in cocultures treated with IL-1β increased ~3-fold compared to cocultures without IL-1β (Figure 3).

TNFRSF11B encodes for the decoy receptor OPG and functions to suppress chondrocyte apoptosis by binding to and suppressing apoptosis in chondrocytes induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Synoviocyte TNFRSF11B expression (P = .04) in IL-1β-stimulated cocultures increased ~10-fold compared to control cocultures (Figure 3). IGFBPs are involved in the transport of IGF into cartilage, and are associated with increased production of proteoglycans by chondrocytes and can inhibit chondrocyte apoptosis. In contrast to the protein abundance ratio, synoviocyte IGFBP2 expression was decreased by...
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~0.2-fold (P = .04) in the presence of IL-1β compared to control cocultures, suggesting that IGFBP2 could be expressed by chondrocytes. Previous studies have shown that A2M can reduce catabolism-associated cartilage damage in vitro and in preclinical post-traumatic OA models through its broad-spectrum ability to block catabolic cytokines and enzymes including IL-1β, thus downregulating the inflammatory cascade.35–38 There was no difference in A2M gene expression between synoviocytes in control cocultures (1.1 ± 1.39) and IL-1β-treated cocultures (0.21 ± 0.08; P = .29).

Figure 2—Analysis of protein–protein interactions by the Search Tool for the Retrieval of Interacting Genes/Proteins. Visualization of network between proteins with an abundance ratio > 1 in coculture media ± interleukin-1β. Colored nodes represent query proteins and the first shell of the interactors. Empty nodes represent proteins of unknown 3-D structure; filled nodes represent some 3-D structure is known or predicted. Edges represent protein–protein associations. A2M = Alpha-2-macroglobulin. AFM = Afamin. C7 = Component C7. CCL16 = C-C motif chemokine 16. CFB = Complement factor B. GSN = Gelsolin. IGF2 = Insulin-like growth factor II. IGFBP2 = Insulin-like growth factor-binding protein 2. ITIH3 = Inter-alpha-trypsin inhibitor. LUM = Lumican. THBS1 = Thrombospondin-1. TIMP3 = Metalloproteinase inhibitor 3 precursor. VCAM1 = Vascular cell adhesion molecule 1. TNFRSF11B = Tumor necrosis factor receptor superfamily member 11B.

Figure 3—Synoviocyte gene expression. Gene expression in synoviocytes from cocultures stimulated with interleukin (IL)-1β was compared to control cocultures using a paired sample t test. A—There was a -3-fold increase in metalloproteinase inhibitor 3 precursor (TIMP3) expression with IL-1β treatment (P = .02). B—Tumor necrosis factor receptor superfamily member 11B (TNFRSF11B) gene expression was increased -10-fold in IL-1β-treated cocultures (P = 0.04). C—Insulin-like growth factor-binding protein 2 (IGFBP2) expression was -0.2-fold decreased in IL-1β-stimulated cocultures (P = .04). Nanogram is a unit of mass; n = 3.
Discussion

This study was based on previous observations that synoviocytes protect cartilage from the catabolic effects of IL-1β in vitro. IL-1β is commonly used in vitro to stimulate cartilage catabolism and inflammation by inducing expression of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and other catabolic enzymes. Similarly to the aforementioned studies, cartilage treated with IL-1β resulted in GAG depletion; however, when in coculture with synoviocytes, GAG was mostly retained, confirming that the synovium secretome has chondroprotective properties against the catabolic effects of IL-1β.

TIMP3 was selected for gene expression analysis because it is an antagonist to many cartilage degradative enzymes such as MMPs, ADAMTS, and aggreganases, and it has been shown to decrease ADAMTS-4, ADAMTS-5, and IL-1α-induced cartilage degradation. The abundance ratio of TIMP3 combined with the gene expression of TIMP3 in synoviocytes from IL-1β treated compared to control cocultures suggests that synoviocytes are synthesizing TIMP3, which could be leveraged for secretome drug development.

The protein TNFRSF11B is also known as OPG. The abundance ratio of TNFRSF11B and gene expression of TNFRSF11B was increased in synoviocytes in IL-1β-stimulated cocultures. This suggests that, like TIMP3, synoviocytes synthesize and secrete OPG. The role of OPG in OA is not completely understood; however, it is known to play important roles in bone turnover, cartilage homeostasis, and the onset of OA. OPG acts as a decoy receptor for the receptor activator of nuclear factor kappa-B ligand, preventing it from binding to the receptor activator of nuclear factor kappa-B on osteoblast precursors and driving their differentiation into osteoclasts. This suggests that aberrant OPG function can affect the balance between subchondral bone formation and resorption, making OPG essential in joint homeostasis.

IGFBP2 and IGFBP4 have chondroprotective properties against the catabolic effects of IL-1β. The use of the proteomic method for determining the mechanisms behind the protective effects provided a comprehensive discovery of known and novel proteins secreted by synoviocytes with anabolic and catabolic properties involved in articular homeostasis. This study could be the starting point for future studies in which the synovium secretome factor could be evaluated in in vitro and in vivo scenarios to assess the to validate the protective roles of the synovium secretome in OA as a disease-modifying product.

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References


**Supplementary Materials**

Supplementary materials are posted online at the journal website: avmajournals.avma.org