Biologic effects of fibrosis-reducing treatment on the spinal cartilage endplate

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INTRODUCTION: Cartilage endplate (CEP) fibrosis is marked by increased amounts of collagen and aggrecan [1], reduced nutrient diffusion to the nucleus pulposus (NP) cells [2] and severer intervertebral disc degeneration [3]. Developing treatments to reduce fibrosis may improve CEP permeability to nutrients, thereby enhancing disc nutrition and regenerative potential. Our previous findings show that treating human CEP tissues with a custom MMP-8 enzyme reduces fibrosis, increases CEP permeability, and improves NP cell survival [4]. An unanswered question is what biologic effects fibrosis-reducing treatments have on the CEP, and specifically, how do the matrix fragments generated from proteolysis impact the CEP cells. For example, matrix fragments could act as damage-associated molecular patterns that activate toll-like receptors (TLRs) and promote a pro-inflammatory response [5]. The goals of this study were to profile the proteins in untreated and MMP-8-treated CEP tissues and to characterize the cellular response to matrix fragments from MMP-8 proteolysis.

METHODS: *Human disc tissues:* Following informed consent, CEP cells and intact CEP biopsies were isolated from surgical waste tissues collected from 5 patients undergoing lumbar fusion (3/2 female/male; 64.6 ± 3.7 years-old). *Mass spectrometry:* We used mass spectrometry to profile the peptides in untreated (n = 4 patients) and MMP-8-treated (n = 2 patients) human CEP biopsies, including the "releasates" from CEP proteolysis by MMP-8 (n = 4 patients). Peptides were analyzed with a TTOF 6600 mass spectrometer (SCIEX) coupled to a nanoflow HPLC (AQUITY UPLC M class) equipped with a nanoEase C18 column (Waters) using mobile phase A (water, 0.1% formic acid) and mobile phase B (acetonitrile, 0.1% formic acid) in conjunction with a linear gradient of 2-40% B over 100 min. Information-dependent acquisition of multiply charged ions with m/z 400-1250 and counts > 150 were subjected to MS/MS, using rolling collision energy and dynamic background subtraction. *Cell culture*: CEP cells from five patients (3/2 female/male; 64.6 ± 4.1 years-old) were expanded to passage 3, and then cultured for 72 hr in four conditions: standard growth medium (negative control); medium supplemented with 100 ng/mL Pam2CSK4 (TLR2/6 agonist, positive control); and medium supplemented with matrix fragments ("releasates") resulting from overnight treatment of patient-matched CEP tissues with 0.2 U/mL (200 ng/mL) or 2.0 U/mL (2000 ng/mL) of recombinant human MMP-8 (Creative Biomart). After culture, conditioned medium was collected, analyzed for cytotoxicity by LDH assay, and assayed by ELISA for MMP-13, MMP-3, CCL5 and IL-6 production. *Statistics:* Spectra were identified with an FDR < 0.05% using all canonical human protein sequences. Analytes were compared using ANOVA with Tukey HSD post-hoc tests.

RESULTS: We identified 8,664 peptides corresponding to 250 unique proteins in the untreated CEP samples, and 5,653 peptides belonging to 201 unique proteins in the treated CEP samples. The exogenous MMP-8 was identified in the treated samples, but not in untreated samples, suggesting good penetration of MMP-8 into the tissue. Present among the proteins discovered **(Fig. 1A)** were known targets of MMP-8, including collagen II (COL2A1). In the releasates from CEPs treated with MMP-8, we identified 288 peptides belonging to 24 unique proteins, with the most abundant protein being collagen II. Also identified in the releasates were two collagen subtypes (COL3A1, COL5A2) that were not identified in the treated or untreated CEP samples. This collagen enrichment suggests productive collagenolytic activity of the enzyme. Finally, of the proteins identified in the untreated CEPs, only 16 (6.4%) were discovered in the releasates following treatment, which supports the specificity of MMP-8 activity with few direct and indirect off-target effects on the target matrix. In response to 72 hr exposure to the releasates from CEP proteolysis by MMP-8, CEP cells exhibited low sensitivity. Cytotoxicity was highest in the TLR2/6 agonist group, but the difference was not significant (5.17 ± 0.18 all groups, p = 0.37). We found minimal effects of the MMP-8 releasates, whereas the TLR2/6 agonist significantly increased production of catabolic factors (MMP-13), inflammatory cytokines (IL-6), and chemokines (CCL5) by the CEP cells **(Fig. 1B-E)**.

DISCUSSION: In many degenerated discs, fibrotic CEPs with excessive amounts of collagen impede nutrient transport into the NP [2]. Here our results showed that treatment of human CEP tissues with recombinant MMP-8 reduces collagen content, and that the matrix fragments from proteolysis by MMP-8 do not promote a short-term pro-inflammatory response at low (0.2 U/mL) or moderate doses (2.0 U/mL). Conversely, CEP cell exposure to a TLR2/6 agonist increased production of inflammatory cytokines and chemokines, which provides indirect evidence for the cells' expression of those TLRs and suggests that matrix fragments from proteolysis by MMP-8 may not activate TLR2/6. Mass spectrometry identified the most abundant proteins in the MMP-8 releasates as collagen II and fibronectin, which confirms the specificity of MMP-8 activity with few off-target effects. We previously found that intradiscal treatment of the CEP with a collagenolytic enzyme improved small-solute transport into human lumbar discs [5]. Taken together, these results suggest that fibrosis-reducing treatments such as MMP-8 may improve CEP permeability to nutrients with minimal short-term, adverse effects on the CEP cells.

SIGNIFICANCE: A key factor in the etiology of disc degeneration and in the poor clinical efficacy of biologic therapies is diminished permeability of the CEP to vital nutrients that sustain the NP cells. Treatment of the CEP with a fibrosis-reducing enzyme, MMP-8, reduces nutrient-blocking matrix constituents and improves NP cell viability. This study provides a critical data about the treatment specificity and biological effects of CEP exposure to MMP-8.

REFERENCES: 1. Antoniou+, Spine 1996; 2. Wong+, Osteoarthritis Cartilage, 2019; 3. Bonnheim+, Eur Spine J 2022; 3. Dolor+, PLoS One 2019; 4. Krock+, Sci Rep, 2017; 5. Habib+, Frontiers in Bioengineering 2023.

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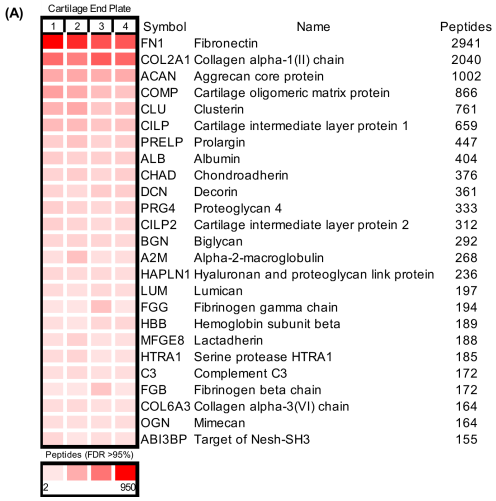

Symbol	Name	Peptides
FN1	Fibronectin	2941
COL2A1	Collagen alpha-1(II) chain	2040
ACAN	Aggrecan core protein	1002
COMP	Cartilage oligomeric matrix protein	866
CLU	Clusterin	761
CILP	Cartilage intermediate layer protein 1	659
PRELP	Prolargin	447
ALB	Albumin	404
CHAD	Chondroadherin	376
DCN	Decorin	361
PRG4	Proteoglycan 4	333
CILP2	Cartilage intermediate layer protein 2	312
BGN	Biglycan	292
A2M	Alpha-2-macroglobulin	268
HAPLN1	Hyaluronan and proteoglycan link protein	236
LUM	Lumican	197
FGG	Fibrinogen gamma chain	194
HBB	Hemoglobin subunit beta	189
MFGE8	Lactadherin	188
HTRA1	Serine protease HTRA1	185
C3	Complement C3	172
FGB	Fibrinogen beta chain	172
COL6A3	Collagen alpha-3(VI) chain	164
OGN	Mimecan	164
ABI3BP	Target of Nesh-SH3	155

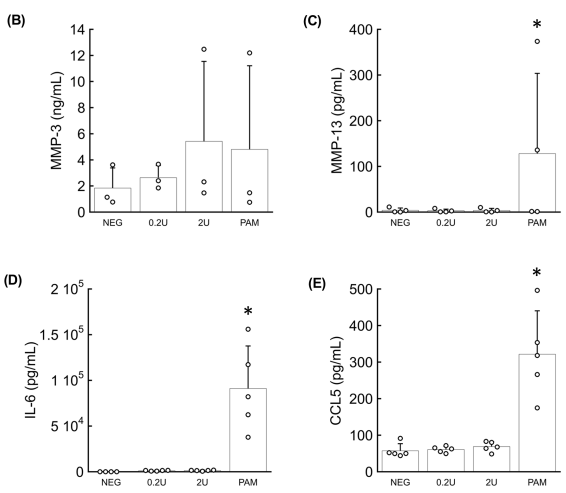

Figure 1: (A) Top 25 proteins identified in untreated CEPs of 4 patients 66-71 yrs old. Effects of matrix fragments by MMP-8 proteolysis on **(B)** MMP-3, **(C)** MMP-13, **(D)** IL-6 and **(E)** CCL5 production by CEP cells. Mean ± SEM for 5 patients (*p < 0.01 vs. negative control). Media contained releasates from proteolysis by MMP-8 (0.2 or 2.0 U/mL) or TLR2/6 agonist Pam2CSK4.