Clinically relevant redifferentiation of fibroblast-like chondrocytes into functional chondrocytes by the low molecular weight fraction of human serum albumin


Abstract

Objective

Traumatic joint injury induces chondrocyte dysfunction and progressive breakdown of articular cartilage, leading to post-traumatic osteoarthritis (PTOA). In this condition, dysfunctional fibroblast-like chondrocytes (FLCs) no longer express proteins required for cartilage maintenance, such as SOX9 and collagen-type II (COL2). Interleukin-6 (IL-6) has been demonstrated to downregulate expression of these two critical proteins in chondrocytes, and increased IL-6 levels have been measured in patients with PTOA. The <5kDa fraction of human serum albumin (LMWF5A) has been suggested to modulate this pathway, as decreased levels of IL-6 are secreted by immunostimulated LMWF5A-treated macrophages. Our objective was to determine whether LMWF5A induces an in vitro model of FLCs to redifferentiate into functional chondrocytes.

Methods

SOX9 and COL2 were monitored via western blot, and COL2 was detected with immunofluorescence. Aggrecan and IL-6 were quantified by ELISA. Glycosaminoglycan (GAG) levels were quantified with alcian blue.

Results

We found that LMWF5A significantly increases the principal cartilage transcription factor SOX9 and the SOX9 target protein COL2 in monolayer-cultured FLCs. Multiple LMWF5A treatments of 3-D pellet FLC cultures over 2wks resulted in a significant decrease in IL-6 and significant increases in the major players of articular cartilage mechanics, aggrecan and highly-sulfated GAGs.

Conclusion

These data support the hypothesis and clinical outcomes of two phase III clinical trials that LMWF5A-treatment induces chondrogenesis and supports functional cartilage. We propose that LMWF5A could maintain articular cartilage integrity in all joints following traumatic injury.

Key words
collagen type II, aggrecans, human chondrocytes, interleukin-6, serum albumin, SOX9 transcription factor, osteoarthritis, glycosaminoglycans
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Introduction
Knee trauma involving the anterior cruciate ligament (ACL) or meniscus is a major risk factor for post-traumatic osteoarthritis (PTOA) and can result in total knee replacement before the age of 50 (1). Upon knee injury, pro-inflammatory cytokines, such as interleukin-6 (IL-6), are released into the synovial fluid (1, 2). Serum levels of IL-6 are associated with knee cartilage loss (3), and in sera from patients with PTOA, IL-6 is significantly increased (2). During OA progression, chondrocytes lose SOX9, a cartilage-specific transcription factor, as well as collagen-type II (COL2) and aggrecan, critical cartilage matrix proteins, thus dedifferentiating into dysfunctional fibroblast-like chondrocytes (FLCs) (4).

We exploited the nature of primary human chondrocytes to dedifferentiate into FLCs over multiple passages in monolayer culture as an in vitro model of cartilage FLCs. The FLC phenotype may develop in part due to IL-6 activation of STAT1/3 leading to decreased extracellular matrix (ECM) with a concomitant decrease in SOX9 expression (5). SOX9 transcriptionally upregulates genes encoding ECM proteins, specifically collagen-type II α 1 chain (Col2a1), which encodes COL2 (6); conversely, Col2a1 is transcriptionally downregulated by IL-6 signalling (7). Previously, we observed up to a 70% decrease in IL-6 in LMWF5A-treated macrophages (8), and herein, we show that LMWF5A-treated FLCs decrease IL-6 and redifferentiate into functional chondrocytes via SOX9 upregulation.

Materials and methods
LMWF-5A production
Ampio Pharmaceuticals (Englewood, CO) produced LMWF5A as described (9).

Cell culture of FLCs
Osteoarthritic human chondrocytes (Asterand, PCD-20-0635), maintained in Dulbecco’s Modified Eagle Medium/ Nutrient Mixture F-12 (DMEM/F12; ThermoFisher) with 20% fetal bovine serum (FBS), were passaged 9-13 times, treating/1wk, fixed with the Image-iT Fixation/Permeabilization Kit, and stained with anti-SOX9 (1:1000, 82630S, Cell Signaling) and anti-rabbit IgG (1:10000, 7074P2, Cell Signaling). Loading controls were either anti-COX IV or anti-histone H3 (1:1000, 5247 or 12648, Cell Signaling); positive control for SOX9 was SW480 lysate (ab3957, Abcam).

COIL immunofluorescence
FLCs treated 2X with aforementioned UNT, SAL, or LMWF5A conditions in T75 flasks were plated at 1x10^5 cells/well into chamber slides (177445, ThermoFisher) for an additional 2d (3X/wk), fixed with the Image-iT Fixation/Permeabilization Kit, and stained with anti-COL2 (ab34712, Abcam) followed by anti-rabbit AlexaFluor488 (A-11034, ThermoFisher), and mounted in media with DAPI (Vector Laboratories). Confocal images were acquired with an Eclipse Ti microscope/C2+ confocal and NIS Elements software (Nikon). Confocal parameters were determined using UNT wells, and all other conditions were taken with those settings. COL2-488 fluorescence intensity was not altered.
COL2 native western blot analysis
Digitonin (1.5% final), G-250 (0.575% final), and native page sample buffer (BN2008, ThermoFisher) were added to protein lysates from 1wk-treated UNT, SAL, or LMWF5A FLCs (Proteome Mammalian Protein kit, Qiagen), separated by blue native page (Novex, ThermoFisher), and subjected to western blotting using anti-COL2 (1:5000, ab34712, Abcam) and anti-rabbit IgG (1:10000, 7074P2, Cell Signaling).

IL-6 and aggrecan quantification
Pellet-conditioned media was snap frozen and stored at -80ºC, then diluted 1:4 for the IL-6 ELISA (3.1-200 pg/mL, BMS213INST, ThermoFisher) and analysed neat for the aggrecan ELISA (10-250 ng/mL, KAP1461, ThermoFisher).

GAG quantification
Pellets were washed with Dulbecco’s PBS, fixed for 15min in 2.5% glutaraldehyde (111-30-8, Electron Microscopy Sciences) diluted in 0.4M MgCl₂ and 25mM sodium acetate (pH 5.6), stained with 500μL 2.5% glutaraldehyde/alcian blue pH 1 (ab150661, Abcam) for 15min at 37ºC, washed 3X with 3% acetic acid, 3X with water, transferred to 96-well plates, and incubated in 100μL 6M guanidine-hydrochloride (G-4505, Sigma) for 48h to release alcian blue. Absorbance was measured at 630nm with a SpectraMax M2 (Molecular Devices). Pellet DNA was purified using the QIAnamp DNA FFPE kit (56404, Qiagen).

Statistical analysis
Graphs represent mean ± standard deviation, and p-values were calculated using a standard student’s two-tailed t-test. p<0.05 was considered significant.

Results
LMWF5A increases SOX9 and COL2 protein in FLCs grown in monolayer
Primary human chondrocytes were passaged in monolayer culture and dedifferentiated into FLCs, as measured by loss of chondrogenic gene expression by QPCR (data not shown). FLCs were either untreated or treated with saline as a control or LMWF5A, protein lysates were harvested over a 24h time course, and western blot analysis was performed to quantify changes in SOX9 (Fig. 1A). While SOX9 was not induced by saline treatment, LMWF5A treatment significantly increased SOX9 protein starting at 4h (Fig. 1B; 3.9-fold vs. SAL; p=0.01) and continuing over the 24h time course (Fig. 1B; 2.5-fold vs. SAL at 24h; p=0.05). SOX9 remained increased in FLCs treated 3X/1wk with LMWF5A versus UNT or SAL-treated FLCs (Fig. 1C and 1D).
SOX9 directly upregulates the Col2a1 gene (6); therefore, we asked whether COL2 increased in LMWF5A-treated FLCs. FLCs treated with LMWF5A 3X/1wk appeared to display larger COL2 immunoreactive aggregates when compared to control conditions (Fig. 1E). To quantify our observations, western blot analysis showed that when compared to UNT controls, LMWF5A significantly increased COL2 protein in FLCs grown in monolayer culture (Fig. 1F; 2.56-fold; *p =0.03).

LMWF5A decreases IL-6 and increases aggrecan and highly-sulfated GAGs in FLCs grown in 3-D pellets

IL-6 is released into synovial fluid upon traumatic joint injury (1), decreases SOX9 and ECM proteins (5, 7), and is significantly decreased in LMWF5A-treated macrophages (8). Considering that we observed increased SOX9 expression in LMWF5A-treated FLCs, we asked whether IL-6 expression decreased in LMWF5A-treated FLCs. We measured IL-6 in pellet-conditioned media and found that multiple LMWF5A treatments over 2 weeks resulted in a significant decrease in IL-6 (Fig. 2B; UNT 330 or SAL 594 vs. LMWF5A 157pg/ml; *p=0.03 and *p=0.001, respectively).

Loss of aggrecan is observed in synovial fluid following joint trauma (11), and considering our observation that LMWF5A treatment of FLCs upregulated SOX9 and that SOX9 transcriptionally upregulates aggrecan expression, we asked whether LMWF5A treatment increases aggrecan. We measured aggrecan in pellet-conditioned media and found that multiple LMWF5A treatments over 2wks results in a significant increase in aggrecan (Fig. 2C; UNT 29 or SAL 19 vs. LMWF5A 74ng/ml; *p=0.03 and *p=8x10⁻⁵, respectively).

Discussion

PTOA develops following joint trauma, and in the case of knee injury, ACL or meniscus involvement is a major predictor of PTOA (1). A therapeutic
advantage of PTOA versus idiopathic OA is that the injury date is known, and early intervention may modify the course of disease, ideally preventing PTOA. Early interventional strategies for PTOA may include targeting inflammatory responses and supporting normal chondrocyte physiology. We propose LMWF5A is an ideal candidate for PTOA prevention because 1) LMWF5A suppresses a pro-inflammatory cytokine associated with PTOA progression, IL-6 (2), in chondrocytes (this study) and macrophages (8), 2) LMWF5A supports functional chondrocytes by inducing SOX9, COL2, and aggrecan, and importantly, these proteins are integrated into ECM that displays increased highly-sulfated GAG composition, which contributes to the biomechanical properties of cartilage, and 3) LMWF5A has been shown to be extremely safe, as it is a derivative of human serum albumin and no adverse drug-related side effects have been reported (13). In addition to beneficial effects on pain and function in OAK patients (14), LMWF5A delays time to total knee replacement in patients with idiopathic OA (15) and may act as a disease-modifying agent, preventing PTOA. Furthermore, all joints can be affected by acute articular cartilage injury; thus, our data support prophylactic treatment with LMWF5A in all joints following traumatic injury for PTOA prevention.

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References