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

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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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Competing interests:
M.A. Hausburg has compensated stock options and owns stock from Ampio Pharmaceuticals.
E.D. Frederick is a compensated employee of Ampio Pharmaceuticals and has compensated stock options from Ampio Pharmaceuticals.
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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, inducing FLC. Before experiments, cells were fed overnight with media containing 10% FBS.

For 24h time course treatment, FLCs ($1x10^5$ cells/well), plated overnight in 500 μL DMEM/F12 containing 10% FBS in 24-well plates, were treated with 500μL media (UNT), saline (SAL) or LMWF-5A for times indicated. For 1wk monolayer treatment, FLCs, were treated with 5% FBS DMEM/F12 (UNT) or a 1:2 dilution of SAL or LMWF5A in 10% FBS DMEM/F12 media. Media was refreshed 3X/wk. Pellet cultures were established according to (10) except that pellets were grown in 1mL of UNT, SAL or LMWF5A conditions (described above). Pellet media were changed 3X/wk for 2wks.

SOX9 western blot analysis

Protein lysates, prepared from untreated FLCs (0h) and treated FLCs at 0.5, 2, 4, 8, 12, and 24h and 1wk, (Qproteome Mammalian Protein kit; Qiagen) according to manufacturer's instructions, were quantitated with Pierce 660nm assay (22662, ThermoFisher), prepared for SDS-PAGE in Bolt Reducing and LDS sample buffers (ThermoFisher Scientific), separated by SDS-PAGE (4-20%), and subjected to western blotting using 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).

COL2 immunofluorescence

FLCs treated 2X with aforementioned UNT, SAL, or LMWF5A conditions in T75 flasks were plated at $1x10^4$ cells/well into chamber slides (177445, ThermoFisher) for an additional 2d (3X treatment/1wk), 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 T*i* 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 (Qproteome 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 $MgCl_2$ 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 QIAamp 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; UNT 868 or SAL 1191 *vs*. LMWF5A 2963 relative density units; $p=0.01$ and $p=0.03$, respectively).

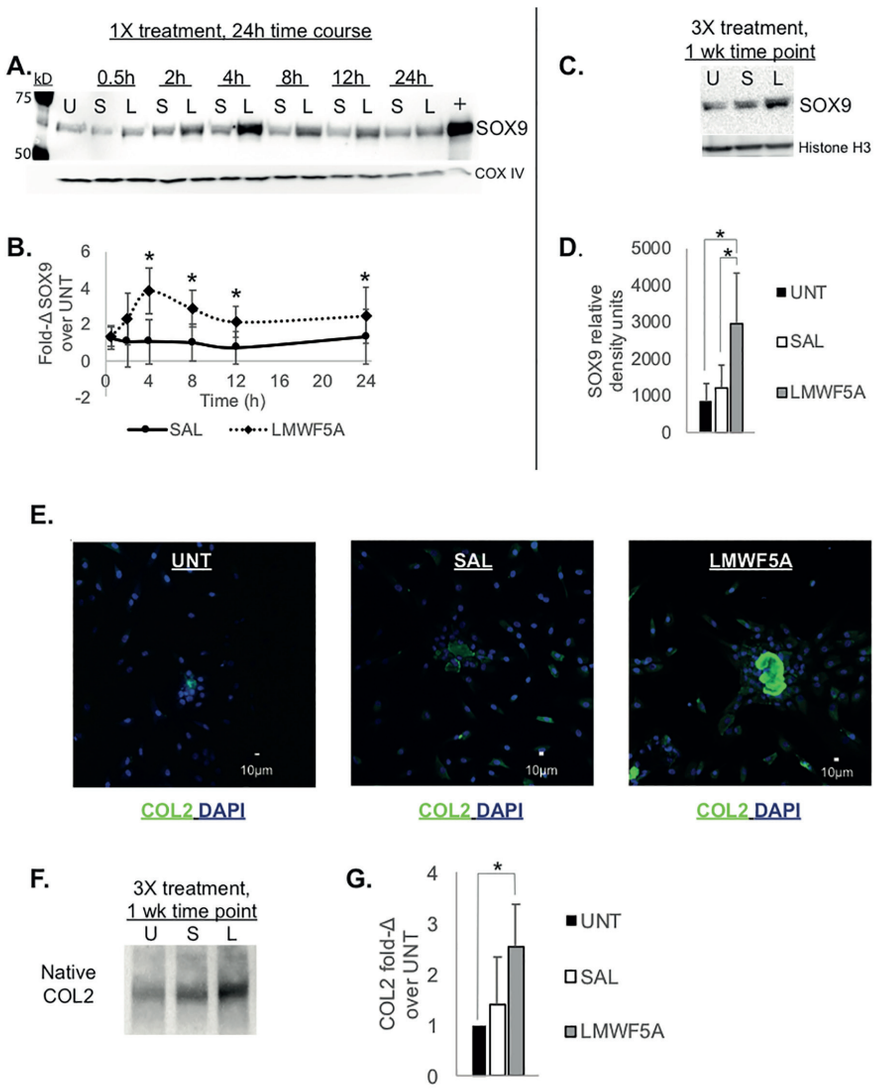

Fig. 1. LMWF5A increases SOX9 and COL2 protein in FLCs cultured in monolayer.
Western blot analysis of SOX9 in protein lysates from untreated FLCs (U) or FLCs treated with saline as a control (S) or LMWF5A (L) once for 0.5, 2, 4, 8, 12, or 24h (**A** and **B**) or three times over one week (**C** and **D**) in monolayer culture. Representative blots are shown in **A** and **C**, and quantification of three independent experiments is shown in **B** and **D**. The fold change in SOX9 expression in **B** was calculated compared to the untreated condition (mean ± SD). * indicates significantly increased SOX9 protein with LMWF5A when compared to saline at that time point ($p<0.05$). Relative density units of the SOX9 bands are displayed in **D** (mean ± SD). * indicates significantly increased SOX9 protein with LMWF5A when compared to untreated or saline conditions ($p<0.05$). **+** indicates SW480 cell lysate used as a control for SOX9 expression. Confocal images of untreated FLCs (UNT) or FLCs treated with saline (SAL) or LMWF5A three times over one week (**E**). COL2 staining is shown in green, and nuclear DAPI staining is shown in blue. Native western blot analysis of COL2 in protein lysates of cells treated as in **E** (**F** and **G**). A representative blot is shown in **F**, and quantification of three independent experiments is shown in **G**. The fold change in COL2 expression was calculated compared to the untreated condition (mean ± SD). * indicates significantly increased COL2 protein with LMWF5A when compared to the untreated control ($p<0.05$).

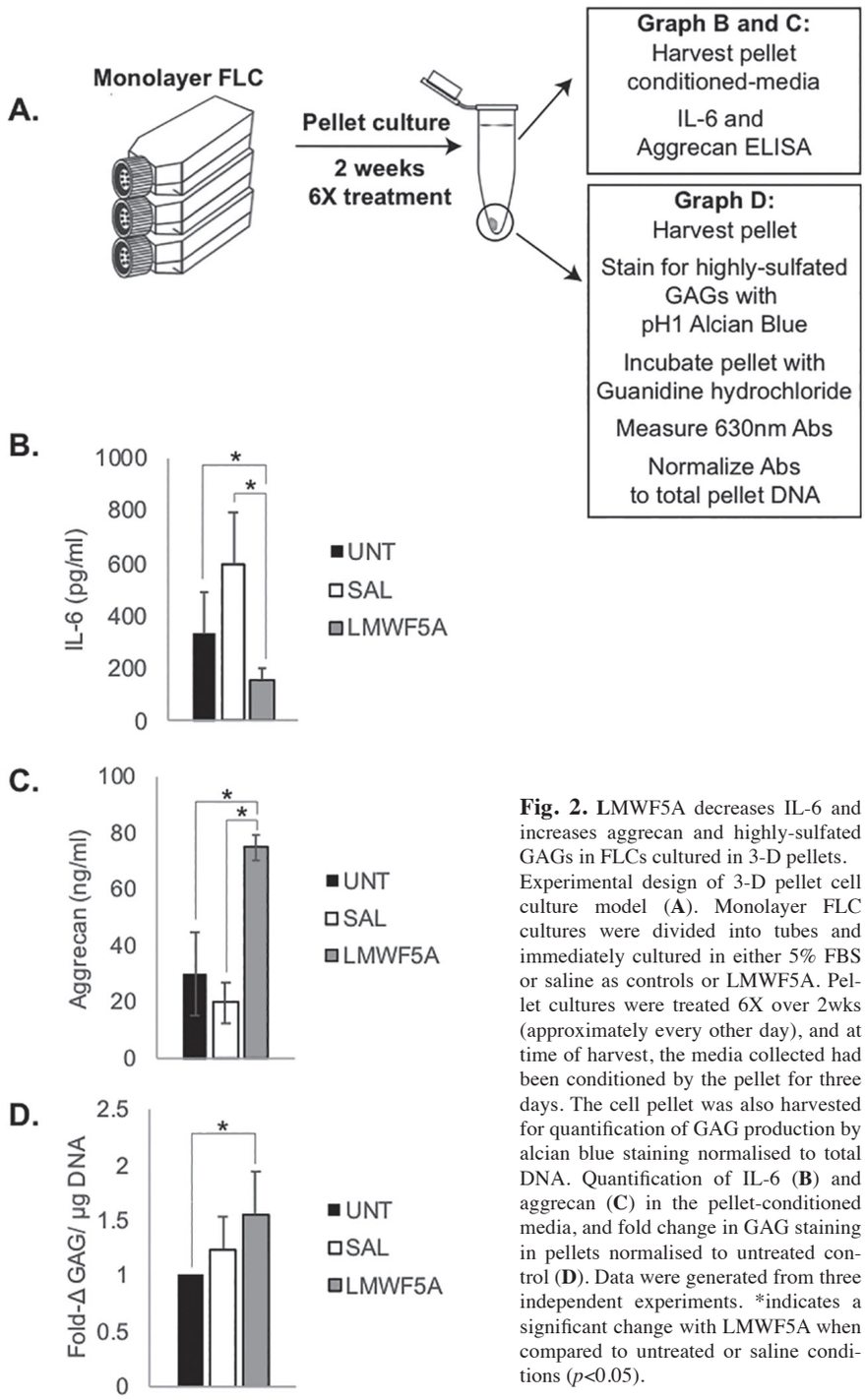

Fig. 2. LMWF5A decreases IL-6 and increases aggrecan and highly-sulfated GAGs in FLCs cultured in 3-D pellets. Experimental design of 3-D pellet cell culture model (**A**). Monolayer FLC cultures were divided into tubes and immediately cultured in either 5% FBS or saline as controls or LMWF5A. Pellet cultures were treated 6X over 2wks (approximately every other day), and at time of harvest, the media collected had been conditioned by the pellet for three days. The cell pellet was also harvested for quantification of GAG production by alcian blue staining normalised to total DNA. Quantification of IL-6 (**B**) and aggrecan (**C**) in the pellet-conditioned media, and fold change in GAG staining in pellets normalised to untreated control (**D**). Data were generated from three independent experiments. *indicates a significant change with LMWF5A when compared to untreated or saline conditions ($p<0.05$).

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 and 1G; 2.56-fold; $p=0.03$).

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

LMWF5A significantly increases critical chondrogenic proteins in FLCs grown in monolayer culture. Thus, we sought to further elucidate the mechanism of action of LMWF5A using 3-D pellet cell cultures, which induce chondrogenesis in FLCs (10) (Fig. 2A).

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 $p=8\text{x}10^{-5}$, respectively).

ECM of cartilage consists of GAGs bound to proteoglycans that stabilise collagens and form a stable matrix that can withstand the biophysical requirements of cartilage. Aggrecan, the major proteoglycan in ECM of cartilage, binds to the highly-sulfated GAGs, chondroitin sulfate and keratin sulfate, which hydrate the matrix allowing for articular cartilage to bear high compressive loads (12). We utilised pH 1 alcian blue, intended for detection of highly-sulfated GAGs, and found that when normalised to pellets cultured in 5% FBS (UNT), the ECM of LMWF5A-treated pellets displayed a significant increase in highly-sulfated GAGs, determined via Abs_{630}/µg DNA (Fig. 1D; 1.6-fold *vs*. UNT; $p=0.02$).

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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