Liver X Receptor activation delays chondrocyte hypertrophy during endochondral bone growth

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Objective: Activation of the Liver X Receptor (LXR) has recently been identified as a therapeutic strategy for osteoarthritis (OA). Human OA articular cartilage explants show decreased LXR expression, and LXRβ-null mice display OA-like symptoms. LXR agonist administration to OA articular cartilage explants suppresses proteoglycan degradation and restores LXR-activated transcription. We aimed to investigate the effect of LXR activation on chondrocyte differentiation to elucidate the molecular mechanisms behind its protection against OA.

Method: The specific LXR agonist, GW3965, was used to examine the effect of LXR activation on chondrocyte differentiation. Tibia organ cultures were used to examine the effect of LXR activation on bone growth and growth plate morphology, followed by immunohistochemical analysis. In ATDC5 and micromass cultures, chondrocyte differentiation was examined through cellular staining and proliferation assays. Various chondrogenic markers were analyzed by real-time reverse-transcription polymerase chain reaction (qRT-PCR) in micromass RNA.

Results: Chondrocyte hypertrophy was suppressed by GW3965 treatment, as shown by decreased hypertrophic zone length in the tibial growth plate, decreased alkaline phosphatase staining in ATDC5 and micromass cultures, and down regulation of Col10a1, Mmp13 and Runx2 expression. Increased proliferation in treated ATDC5 cells and up-regulation of Col2a1 expression in treated micromass cultures suggest hypertrophy is suppressed secondary to prolonged proliferation. Decreased p57 levels in treated growth plates suggest this to be due to cell-cycle exit delay.

Conclusion: Our findings regarding LXR’s role in cartilage development provide insight into how LXR activation prevents cartilage breakdown, further solidifying its potential as a therapeutic target of OA.

Introduction

Longitudinal bone growth in the body is attributed to the activity of the growth plate in the process of endochondral ossification, in which a cartilage template intermediate is formed and then replaced by bone specific matrix. Endochondral ossification begins with the condensation of mesenchymal cells, which then initiate chondrogenic differentiation under the control of the transcription factor Sox9. These chondrocytes first proliferate while producing extracellular matrix rich in collagen II and proteoglycan (aggrecan), then exit the cell cycle and undergo hypertrophy starting from the center of the cartilage anlage. The hypertrophic chondrocytes initiate mineralization of their matrix and undergo apoptotic cell death. This allows the invasion of blood vessels, osteoblasts, and osteoclasts to remove the cartilage matrix for the deposition of bone matrix, ultimately forming the primary ossification center. Postnatally, chondrocytes at the two distal ends of the bone undergo a similar differentiation process to form the secondary ossification centers. The developing cartilage that remains between the primary and the secondary ossification centers is defined as the growth plate, consisting of distinct cell layers of resting, proliferative and hypertrophic zones. While many markers of chondrocyte differentiation are known, many nuclear factors and signaling molecules regulating this process remain to be elucidated.

Certain characteristics of osteoarthritis (OA) have been found to resemble the endochondral ossification process. As opposed to growth plate chondrocytes that are much more dynamic, healthy articular chondrocytes remain in a stable state and do not differentiate further. However, osteoarthritic articular chondrocytes lose their stable state and can initiate behavior similar to growth plate chondrocytes, such as proliferation, terminal differentiation.
and hypertrophy, and apoptosis\textsuperscript{9–12}. Furthermore, proteolytic enzymes such as matrix metalloproteinase (MMP)-13 and aggrecanases produced by hypertrophic chondrocytes to degrade their surrounding matrix within the growth plate are also produced in OA cartilage\textsuperscript{13}. As such, it is reasonable to postulate that signaling molecules involved in the pathogenesis of OA may also be involved in regulating chondrocyte differentiation of the growth plate\textsuperscript{14,15}.

The Liver X Receptors (LXRs)/NR1H3 and LXRs/NR1H2 are nuclear receptors best known for their central roles in regulation of lipid metabolism and inflammatory signaling\textsuperscript{16–18}. However, recent studies have suggested LXR to possess roles in cartilage that offer protection against OA. LXR\texttextsuperscript{b-null} mice display OA-like symptoms, including proteoglycan degradation and prostaglandin-E2 production in the articular cartilage\textsuperscript{19}. Furthermore, human OA articular cartilage explants exhibit decreased LXRs and LXR\texttextsuperscript{b} expression\textsuperscript{19,20}. Interestingly, the administration of synthetic LXR agonists to human OA articular cartilage explants suppresses proteoglycan degradation and restores LXR-activated transcription\textsuperscript{19,20}. However, the cellular and molecular mechanisms underlying LXRs protective role in cartilage are not completely understood.

In this study, we hypothesize that LXR plays a role in the regulation of chondrocyte differentiation, specifically by controlling chondrocyte hypertrophy. To test our hypothesis, the specific LXR\texttextsuperscript{c} agonist, GW3965, was used to examine the effect of LXR activation on different culture systems. Markers of chondrogenic differentiation were subsequently analyzed at the mRNA and protein level to help elucidate underlying mechanisms. Our findings show that LXR activation using a synthetic agonist results in enhanced chondrocyte proliferation and reduced chondrocyte hypertrophy. These results suggest LXR’s role in regulating enchondral ossification to be a possible mechanism in protection against OA.

Materials and methods

Timed pregnant CD1 mice were purchased from Charles River Laboratories (St. Constant, QC). All procedures involving mice were conducted following the animal protocol approved by the University of Western Ontario Animal Care and Use Committee. All organ culture and cell culture media components were from Sigma–Aldrich (Oakville, ON), Gibco (Life Technologies Inc, Burlington, ON), and Invitrogen (Life Technologies Inc, Burlington, ON) unless otherwise stated. The LXR agonist, GW3965, was purchased from Sigma–Aldrich. All real-time PCR reagents and probes were purchased from Applied Biosystems (Life Technologies Inc, Burlington, ON).

Tibiae organ culture

Tibiae were isolated from E15.5 CD1 mice and cultured in media as previously described\textsuperscript{19,21}. Tibiae were then incubated for 6 days. Treatment commenced the day following tibia isolation (day 1) using dimethylsulfoxide (DMSO) (Sigma–Aldrich) as the vehicle, and the LXR agonist GW3965 at concentrations of 1 μM, 2 μM, 5 μM and 10 μM. Measurements of bone length were taken on day 1 prior to treatment using both the Zeiss Stemi SV6 Stereo Zoom microscope with eyepiece, as well as the Leica EC3 microscope and Leica Application Suite V3 Software (Leica Microsystems Inc, Concord, ON). Media with treatment were changed every other day, and after 6 days another measurement was taken. The calculated difference in tibia length between time-points was used as a measure of bone growth.

Histology

Tibiae explants were rinsed with phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde (PFA) (Sigma–Aldrich). Tibiae were then marked with 2% mercureochrome, placed in 70% ethanol, and sent for embedding and sectioning at the Robarts Research Institute, Molecular and Pathology (London, ON). Safranin-O/Fast Green staining was performed as described with minor modifications\textsuperscript{22}. After dewaxing in xylene and rehydration in a series of graded alcohol washes, sections were incubated in hematoxylin for 2 min, 0.02% Fast Green (Harleco, Darmstadt, Germany) for 25 min followed by 7 dips in 1% acetic acid solution, and finally 0.1% Safranin-O (Sigma–Aldrich) for 7 min. Slides were then dehydrated and mounted using a xylene-based mounting media. Histological quantification was performed on stained sections through the measuring of the length of individual growth plate zones as well as the total growth plate. On all sections, the length of the total growth plate was first identified, followed by the hypertrophic zone and the proliferative and resting zones collectively. All measurements were done using the distance line tool on Leica Application Suite V3 Software.

Immunohistochemistry (IHC)

Paraffin sections were dewaxed and rehydrated followed by incubation in 3% H2O2. Sections were then boiled in sodium citrate (pH 6.0), blocked with 5% goat serum in PBS, and incubated with Kip2/p57 primary antibody (sc-8298, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight with secondary antibody subsequently applied\textsuperscript{23,24}. After colorimetric detection with diaminobenzidine (DAB) (Dako, Markham, ON) substrate solution, sections were counterstained with methyl green and mounted. Quantification of IHC was performed by counting all positively stained (brown) cells within a 58.48 × 107.53 μm area of interest. All values presented were absolute numbers of positively stained cells in control or GW3965-treated growth plates. All images were captured using the Leica DFC295 microscope and Leica Application Suite V3 Software.

ATDC5 cell culture

ATDC5 cells were cultured and induced to differentiate with 1% insulin–transferrin–selenium (ITS) (Sigma–Aldrich) the day after plating as described\textsuperscript{19,21}. Cells were plated at a density of 2 × 10\textsuperscript{4} cells/ml in 24-well plates for a period of 18 days. Treatment was commenced on day 3 with DMSO as the vehicle or 1 μM GW3965. Media including treatment were changed every other day throughout the entire time-course.

Proliferation assays

Thiazolyl blue tetrazolium bromide (MTT) proliferation assay was performed as described with minor modifications\textsuperscript{15,28}. ATDC5 cells were plated at 2 × 10\textsuperscript{4} cells/ml in 24-well plates. Cells were treated with DMSO or 1 μM GW3965 for a period of 15 days, with media changed every other day. On days 3, 6, 9, 12, and 15, 1:10 dilution of 5 mg/mL MTT (Sigma–Aldrich) in PBS was added to cells and incubated for 4 h at 37°C. Solubilization using acidified isopropanol (0.04 M HCl in absolute isopropanol) followed, and the converted dye was quantified using a Safire plate-reader (Tecan, Mannedorf, Switzerland) and XFluor4 software at 570 nm with background subtraction at 650 nm. The absorbance (A\textsuperscript{opt700nm}−A\textsuperscript{opt500nm}) correlates directly to living cell number in ATDC5 cells.

To assess total cell number, direct cell counting was performed. ATDC5 cells plated at the same density were trypsinized for 2–5 min. After neutralizing with media, cell solution of each well was plated and counted on each day of the time-course.
Micromass culture

Limb buds (forelimb and hindlimb) of E11.5 CD1 mouse embryos were isolated, then dissociated in a solution containing puck saline A (PSA), 10% (v/v) chick serum, and 2% (v/v) dispase (50 caseinolytic units/ml, BD Biosciences, San Jose, CA) for 1.5 h at 37°C and 100 rpm. The dispase activity was neutralized and a single cell suspension was extracted as described29,30. After cell counting and a brief centrifugation, cells were resuspended in micromass media at a concentration of 2.5 × 10^6 cells/ml and plated into six-well Nunc cell culture plates, at eight 10^5 cells/well. Plated cells were incubated at 37°C and 5% CO2 for 1 h to allow cell adherence, followed by the addition of 2 ml/well of micromass media supplemented with 10 mM β-glycerophosphate, 50 μg/ml ascorbic acid, and the treatment of DMSO or GW3965. Cells were cultured for a period of 15 days, with media and treatment replenished daily.

Cell staining

Micromass cultures were stained with alcian blue as described29. Both micromass cultures and ATDC5 cell cultures were stained for alkaline phosphatase (ALP) activity as previously described, with minor modifications29,30. Cells were washed twice with cold PBS, then suspended in radioimmunoprecipitation assay (RIPA) buffer and harvested. Following 30 min of incubation on ice, cell lysates were centrifuged for 25 min at 4°C at 14,000 rpm. The supernatant was collected and protein concentration was quantified using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s instructions. Subsequently, 19 μg of protein was loaded and separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by protein transfer onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was then blocked with tris buffered saline-tween with 5% bovine serum albumin (5% BSA-TBST), and incubated with primary antibody against p57/Kip2 (ab75974, Abcam, Cambridge, United Kingdom) and β-actin (A5441, Sigma–Aldrich). After washing in TBST, the membrane was incubated with goat-anti-rabbit (sc-2004, Santa Cruz), or goat-anti-mouse (sc-2005, Santa Cruz) immunoglobulin G-horseradish peroxidase conjugated secondary antibody. Signal visualization was performed using the Clarity Western ECL Substrate (Bio-Rad) and the Bio-Rad ChemiDoc MP Imaging System.

Statistical analysis

All data collected were from at least three independent trials. Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using IBM SPSS version 22.0 (Armonk, NY). One-way analysis of variance (ANOVA) with the Tukey’s post-hoc test was performed to identify significant differences among groups. P-values < 0.05 were considered statistically significant.

Results

LXR activation alters growth plate morphology ex-vivo

To investigate the effect of LXR activation on longitudinal bone growth, E15.5 tibiae were isolated and cultured in the presence of a specific LXR agonist, GW3965. Tibiae treated with varying concentrations of GW3965 (0, 1, 2, 5 or 10 μM) did not reveal significant changes in growth, although growth appeared to be decreased in all treated tibiae compared to the control (Fig. 1). To further examine how GW3965 treatment affected E15.5 tibial growth plates, histological analysis was performed using Safranin-O/Fast Green staining [Fig. 2(A)]. Quantitative measurements of individual growth plate zones demonstrated the hypertrophic zone to be significantly reduced in length when treated with 1 μM, 5 μM and 10 μM GW3965 [Fig. 2(B)]. The length of the total growth plate and the proliferating and resting zones combined remained comparable between treated and control conditions in the 0, 1, 2, 5 and 10 μM GW3965 treated groups.

Fig. 2. Histological analyses of GW3965-treated tibia organ cultures. (A) After 6 days in culture with DMSO as vehicle or different concentrations of GW3965, tibiae were embedded and sectioned. Sections were stained with Safranin-O/Fast Green to visualize growth plate morphology. Representative images show the different zones of the growth plate (R+P – resting and proliferating zones, H – hypertrophic zone) (n = 6). Scale bar = 200 μm. (B) Histological quantification was conducted on stained sections by measuring individual growth plate zones. The values are presented as the mean of six independent trials, with 95% CI.
and control tibiae [Fig. 2(B)]. These collective results suggest that LXR activation suppresses chondrocyte hypertrophy.

The cell-cycle exit marker p57 is decreased in GW3965-treated tibiae

In order to gain insight into the mechanisms underlying the shortened hypertrophic zones in GW3965-treated tibiae, the cell-cycle exit marker p57 was studied using IHC [Fig. 3(A)]. Quantification of p57 IHC demonstrated that the number of positively stained cells in GW3965-treated growth plate decreased relative to control [Fig. 3(B)]. This demonstrates that the suppression of chondrocyte hypertrophy observed in the tibial growth plate is in part due to a delay in cell-cycle exit.

LXR activation results in decreased hypertrophy and increased proliferation in ATDC5 cells

To further assess the role of LXR activation in endochondral bone growth and development, ATDC5 cell cultures were used to analyze chondrogenic differentiation over a continuous time-course. Staining for alkaline phosphatase, a marker of chondrocyte hypertrophy, was markedly decreased upon treatment with 1 μM GW3965 [Fig. 4(A)]. This reduction in staining was evident as early as day 9 and persisted throughout the time-course [Fig. 4(A)]. Next, the effect of GW3965 on proliferating chondrocytes was analyzed using the MTT assay and direct cell counting. Our previous data had shown that MTT values in ATDC5 cells and primary chondrocytes directly correspond to cell numbers25,28. These methods show cell number to consistently increase with GW3965 treatment [Fig. 4(B and C)], suggesting the suppression of chondrocyte hypertrophy by GW3965 to be secondary to prolonged proliferation.

LXR activation delays chondrocyte differentiation in micromass cultures

We next used a micromass culture system consisting of mouse embryonic mesenchymal cells plated in high-density droplets. Compared to ATDC5 cells, this ex-vivo system is a more physiological model of chondrogenesis. The amount of alkaline phosphatase staining was reduced in GW3965-treated micromasses [Fig. 5(A)]. This reduction appeared to be more dose-dependent than that shown by alcian blue [Fig. 5(B)]. Alcian blue, a stain that detects the presence of glycosaminoglycans, was decreased upon treatment of 1 μM GW3965, and slightly more so with 5 μM GW3965 [Fig. 5(B)]. Quantification using the Leica Application Suite V3 Software demonstrated area of positive alcian blue staining to decrease with GW3965 treatment [Fig. 5(C)]. For both stains, differences between control and treatment became especially apparent starting on day 6 and persisted throughout the time-

Fig. 3. Immunohistochemical analyses of p57 protein localization in GW3965-treated tibiae. E15.5 tibia organ cultures were cultured for 6 days with DMSO or various concentrations of GW3965, then fixed and prepared for histology. Incubation of primary and secondary antibodies was followed by colorimetric visualization with DAB substrate. (A) Representative images were shown from four independent experiments. Scale bar = 200 μm. (B) Quantification of p57 IHC was performed by counting the number of positively stained (brown) cells within a pre-set box centered on the pre-hypertrophic zone. Data are presented as the mean of three independent trials, with 95% CI.
course. These ex-vivo results further validate the effect of LXR activation on suppression of chondrocyte hypertrophy, and interestingly shows proteoglycan content to also be affected.

LXR activation down-regulates gene expression of late-stage chondrogenic markers in micromass cultures

To better understand the mechanisms underlying the effect of GW3965 on chondrogenic differentiation, real-time reverse-transcription PCR was performed to examine the expression of various genes involved in cartilage development. This analysis was performed using RNA extracted from micromass cultures treated with DMSO or GW3965 (1 or 5 μM). Of the early chondrogenic markers analyzed, mRNA levels of Col2a1 significantly increased with GW3965 treatment starting on day 9, while Sox9 and Aggreca expression remained unchanged [Fig. 6(A)]. Chondrocyte hypertrophy markers Runx2, Col10a1 and Mmp13 all significantly decreased upon treatment with GW3965 on day 12 of culture [Fig. 6(B)]. Pthlh and Ii1h, major regulators of chondrocyte proliferation and hypertrophy, decreased with treatment throughout the
Fig. 5. Chondrogenic differentiation of micromass cultures with LXR activation. E11.5 mouse mesenchymal limb bud cells were plated in micromass cultures and cultured for 15 days in the presence of either DMSO or GW3965. For all staining, at least three independent experiments were used for each time-point, and representative images are shown. (A) Alkaline phosphatase staining was performed every 3 days starting on day 3 to examine chondrocyte hypertrophy. Scale bar = 1 mm. (B) Alcian blue content was examined every 3 days starting on day 3 to assess proteoglycan content and early chondrogenesis. Scale bar = 1 mm. (C) Alcian blue content was quantified using the Leica Application Suite V3 Software, where thresholds were chosen for each time-point using the control image of that time-point. This threshold was then applied to all treatments to determine the total area of positive staining. Values are represented as the mean with 95% CI (n = 3).
Fig. 6. Analyses of chondrogenic gene expression in micromass cultures with LXR activation. Micromass cultures composed of E11.5 mouse limb bud mesenchymal cells were cultured for 15 days and treated with different concentrations of GW3965. RNA was isolated every 3 days starting on day 3. Gene expression was analyzed by qRT-PCR and normalized to Gapdh. Data were expressed relative to values of day 3 control, and are represented as the mean with 95% CI.

(A) The mRNA expression levels of early chondrogenic markers Sox9, Col2a1, and Acan (n = 4 or 6).

(B) The mRNA expression levels of terminal chondrogenic markers Runx2, Col10a1, and Mmp13 (n = 4).

(C) The mRNA expression levels of chondrocyte proliferation and hypertrophy regulating factors, Cdkn1c, Ihh, and Pthlh (n = 4).
time-course and on day 12 of culture, respectively [Fig. 6(C)]. Although the expression of Cdkn1c demonstrated a slight increase on day 12 with GW3965 treatment [Fig. 6(C)], both mRNA and protein levels of p57 remained unchanged in ATDC5 cells upon treatment (Supplementary Fig. 1). These results show that effects of LXR activation on chondrocyte gene expression are consistent with its effects on histological parameters, with the exception of p57, potentially demonstrating context-specific expression patterns.

**Discussion**

Several nuclear receptors have been identified and shown to be involved in endochondral ossification through its implications on Sox9 expression and other targets, including estrogen-related receptors (ERRs), retinoid acid receptors (RARs) and peroxisome proliferator-activated receptor γ (PPARγ)52-55. However, much less is known about the role of LXR. This study aimed to determine whether LXR actively regulates the different stages of chondrogenic differentiation. We used a synthetic LXR agonist, GW3965, to study the specific effects of LXR activation on cartilage development.

The most prominent effect of LXR activation was the decrease in chondrocyte hypertrophy observed in three different chondrocyte culture systems. Ex-vivo tibia organ cultures treated with GW3965 showed a significant decrease in hypertrophic zone length. Similarly, alkaline phosphatase staining was markedly decreased upon LXR activation in both micromass cultures and in ATDC5 cell cultures. Moreover, the mRNA levels of major chondrocyte hypertrophy markers Col10a1, Mmp13, and Runx2 were all decreased with LXR activation. As such, LXR appears to be a negative regulator of chondrocyte hypertrophy. As chondrocyte hypertrophy is one of the largest contributors to skeletal growth30, GW3965’s ability to suppress hypertrophy also explains why a trend towards a decrease in bone growth was observed with treatment.

The observed decrease in hypertrophic zone length can be speculated to be the result of a few possible mechanisms. Terminal chondrogenic differentiation could accelerate, and thus prompt faster turnover of hypertrophic cartilage to bone31. Alternatively, overall differentiation could decrease, resulting in the shortening of each zone31. Our data from proliferation assays (both MTT assays and cell-counts) conducted on ATDC5 cells suggest another mechanism whereby hypertrophy is delayed through prolonged proliferation. Although MTT proliferation assay results can only act as correlates to cell proliferation by delaying cell-cycle exit. Further investigation of the direct targets of LXR activation provides new insights into the regulation of hypertrophic differentiation. In particular, LXR activation suppresses chondrocyte hypertrophy through the prolongation of cellular proliferation by delaying cell-cycle exit. Future studies integrating chromatin immunoprecipitation (ChIP) sequencing and gene expression analyses will help identify LXR target genes in cartilage and elucidate underlying mechanisms.

In summary, our study demonstrates the novel finding that LXR activation protects from OA progression18,19, but the mechanisms responsible for this protective effect were not completely understood. Here we show that suppression of hypertrophic differentiation of articular chondrocytes, which promotes OA progression13,14, is one such mechanism. Future studies will have to address the direct target genes of LXR transcription factors in chondrocytes. Genome-wide profiling of LXR targets has thus far been performed in liver cells, macrophages and foam cells44-46. Among the new target genes of LXRα identified by Feldmann et al. in foam cells is PARP-1, which encodes for the enzyme Poly(ADP-ribose) polymerase-1. This enzyme is a ubiquitous nuclear enzyme that is implicated in various biological processes, including DNA damage repair47. Knockdown of PARP-1 has also shown reduced cell proliferation in a human embryonic kidney cell line48. These findings suggest that PARP-1 could be a candidate gene up-regulated by LXR activation in chondrocyte differentiation. Future studies integrating chromatin immunoprecipitation (ChIP) sequencing and gene expression analyses will help identify LXR target genes in cartilage and elucidate underlying mechanisms.

In our study, the novel finding that LXR activation by its synthetic agonist, GW3965, affects the regulatory processes of chondrocyte differentiation. In particular, LXR activation suppresses chondrocyte hypertrophy through the prolongation of cellular proliferation by delaying cell-cycle exit. Further investigation of the direct targets of LXR will shed light on how LXR regulates this process. The ability of activated LXR to delay hypertrophic differentiation might be a useful attribute in the development of disease-modifying drugs for OA treatment.

**Author contributions**

M.M.S. participated in designing the study and carried out the experiments, as well as collected, analyzed and interpreted the data. F.B. conceived the study design, and coordinated and oversaw the study and data interpretation. Both authors drafted the manuscript and participated in the final approval of the article. M.M.S. (msun33@uwo.ca) and F.B. (fbeier@uwo.ca) are responsible for the integrity of this work.

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**Conflict of interest**

None to declare.
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Supplementary data

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