Halofuginone attenuates articular cartilage degeneration by inhibition of elevated TGF-β1 signaling in articular cartilage in a rodent osteoarthritis model

WENBO MU1, BOYONG XU1, HAIRONG MA1,2, BAOCHAO JI1, ZHENDONG ZHANG1, JIAO LI1, ABDUSAMI AMAT1 and LI CAO1

Departments of 1Orthopaedics and 2Clinical Medical Research Institute, First Affiliated Hospital of Xinjiang Medical University, Urumqi, Xinjiang 830054, P.R. China

Received January 24, 2017; Accepted August 14, 2017

DOI: 10.3892/mmr.2017.7549

Abstract. Osteoarthritis (OA) is the most common degenerative condition of the weight-bearing joints worldwide without effective medical therapy. In order to investigate whether administration of halofuginone (HF) may attenuate OA, the present study allocated 3-month-old male mice into Sham group, vehicle-treated anterior cruciate ligament transection (ACLT) group and HF-treated ACLT group. The present study determined that HF treatment reduced the expression of matrix metallopeptidase-13 and collagen X in articular cartilage. Additionally, it lowered the Osteoarthritis Research Society International-Modified Mankin score and prevented the loss of articular cartilage from Safranin O and Fast Green staining. HF reduced the progression of osteoarthritis by downregulating abnormally elevated TGF-β1 activity in articular cartilage. Administration of HF may be a potential preventive therapy for OA.

Introduction

Osteoarthritis (OA) is the most common type of arthritis, characterized by pain and loss of joint function (1). Knee joint OA has been reported to affect 3.64% of the population worldwide in 2010 (2). Currently, there is no effective disease-modifying therapy for OA. Pain management and joint replacement are the options for end-stage OA (3,4). The pathogenesis of OA remains unclear and an improved understanding is required in order to target the prevention and effective intervention for early-stage OA (5).

Key words: osteoarthritis, articular artilage, TGF-β1, elevation, halofuginone

Materials and methods

Correspondence to: Dr Li Cao, Department of Orthopaedics, First Affiliated Hospital of Xinjiang Medical University, 137 South Liushan Road, Urumqi, Xinjiang 830054, P.R. China
E-mail: xjbone@21cn.com

Cell culture. The ATDC5 murine chondrogenic cell line (Riken Cell Bank, Tsukuba, Japan), were plated at a density of 1.2x10^4 cells/cm^2 in 6-well plate (Corning, Inc., Corning, NY,
USA). The cells were cultured with maintenance medium, which was Dulbecco's modified Eagle's medium/F12 (11320-033; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 5% fetal bovine serum (10099-141; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (SV30010; GE Healthcare Life Sciences, Logan, UT, USA), at 37°C in a humidified atmosphere of 5% CO2. The maintenance medium and it was exchanged every other day. The differentiation medium, with 1% Insulin-Transferrin-Selenium (41400-045; Gibco; Thermo Fisher Scientific, Inc.) was added to the maintenance medium, which was used when the cells became confluent. The differentiation medium was changed every other day.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). ATDC5 cells were cultured with differentiation medium for 7, 14 and 21 days. Total RNA was extracted using TRIzol reagent (15596026, Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized using PrimeScript RT Master mix (RR036A; Takara Bio, Inc., Otsu, Japan). Three or more different samples were used. qPCR reactions were performed using SYBR Fast qPCR mix (RR430A, Takara Bio, Inc.) according to the manufacturer's protocol. Amplification curves of samples were converted into relative expression values according to the curve of standard controls. Relative quantification of each gene was normalized against GAPDH. The 2−ΔΔCq method was used to calculate relative gene expression levels (20). The sequences of the following primers were used: GAPDH forward (F) 5'-AGCTTCGGCCACATATTCTACCTG-3' and reverse (R) 5'-GGTCACTCTCCATGCAAACA-3'; Col II F 5'-ACGAACCGCGCTGGAACACCTA-3 and R 5'-CCCCCTGGCCCTCATTCTCTCATCA-3; Col X F 5'-TGCCGTGCTGTCTGTCTTCTAC-3' and reverse (R) 5'-TCAAATGGGATGGGGGCACCTACT-3. Amplification cycle was set as 94°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 60°C for 10 sec, and finally a melt curve was inserted with 65°C to 95°C.

Cell Counting Kit-8 (CCK-8) analysis. The toxicity of HF on chondrocytic ATDC5 cells was observed using the CCK-8 method. After 14 days of chondrogenic differentiation, ATDC5 cells were seeded in 96-well plates with a density of 4x10³ cells/well and incubated for 24 h. The cells were treated with different concentrations of HF (0, 6.25, 12.5, 25, 50, 100 and 200 ng/ml) CCK-8 (CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was applied to monitor cell viability after 24, 48 and 72 h and the percentage of cell viability was calculated based on the absorbance at 450 nm.

Western blot analysis. After 14 days of chondrogenic differentiation, ATDC5 cells were treated with HF (17395-31-2; Watson Noke Scientific Ltd., Kunshan, China) at different doses 0, 6.25, 12.5 and 25 ng/ml for 6 h, and were treated with 2 ng/ml rhTGF-β1 (240-B-002; R&D Systems, Inc., Minneapolis, MN, USA) for 30 min and processed for western blotting. Then the time-course experiment was performed. After 14 days of chondrogenic differentiation, ATDC5 cells were treated without HF for 0 h, or with 25 ng/ml HF for 4, 8 and 24 h. Then cells were treated with either 2 ng/ml rhTGF-β1 or the reagent used to reconstitute rhTGF-β1 (sterile 4 mM HCl containing 1 mg/ml bovine serum albumin) in an equal volume for 30 min and processed for western blotting. Samples (20 μg) were subjected to 10% SDS-PAGE (4561083; Bio-Rad Laboratories Inc., Hercules, CA, USA) and transferred onto polyvinylidene fluoride (PVDF) membranes (ISEQ0010; Merck KGaA, Darmstadt, Germany). Membranes were blocked in 5% bovine serum albumin (AD0023; Sangon Biotech Co., Ltd., Shanghai, China) for 1 h, incubated with primary antibodies against Smad2/3 (1:1,000; cat. no. 3102S; Cell Signaling Technology Inc., Danvers, MA, USA), phosphorylated (p)-Smad2/3 (1:1,000; cat. no. 3108; Santa Cruz Biotechnology Inc., Dallas, TX, USA) and GAPDH (1:1,000; cat. no. 21188; Cell Signaling Technology Inc.) at 4°C overnight, followed by incubation with secondary antibody (peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L); 1:5,000; cat no. ZB-2301; OriGene Technologies, Inc., Beijing, China) for 2 h. Protein was visualized using Pierce Fast Western Blot kit, enhanced chemiluminescence substrate (cat. no. 35055; Thermo Fisher Scientific, Inc.).

Experimental animals. C57BL/6 male mice (3-month old, 24-25 g, 22 in total) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were maintained in an animal room on a 12-h light/dark cycle with a temperature and humidity of 25±2°C and 55%, respectively. The mice were provided with food and water ad libitum. The anterior cruciate ligament of the right knee was transected to establish a destabilized OA animal model. Sham operation was performed on independent mice by opening the knee joint capsule and suturing the incision in the right knee joint. Mice were divided into Sham, vehicle-treated anterior cruciate ligament transection (ACLT) group and HF-treated ACLT group (n=6-8 per group). Either HF (0.25 mg/kg) or distilled water of equivalent volume (0.2 ml) was administered by oral gavage every other day for 30 days since the second day post-operation. Mice were sacrificed at day 30 post-operatively. All animal experiment protocols were reviewed and approved by the Institutional Animal Care and Use Committee of First Affiliated Hospital of Xinjiang Medical University (Xinjiang, China).

Histochemistry, immunohistochemistry and histomorphometry. When the mice were sacrificed, the right knee joints were resected and fixed in 10% buffered formalin for 24 h. The specimen was decalcified in 10% EDTA (pH 7.3) for 21 days and embedded in paraffin sagittally. Serial sections at 4-μm thickness were incubated with primary antibodies against MMP-13 (1:100; cat. no. ab39012; Abcam; Cambridge, UK), Col X (1:100; cat. no. ab58632; Abcam), TGF-β1 (1:100; cat. no. ab92486; Abcam) and p-Smad2/3 (1:40; cat. no. sc-11769; Santa Cruz Biotechnology Inc.) overnight at 4°C. Subsequently a horseradish peroxidase-streptavidin detection system (1:1,000; cat. no. ZB-2301 and ZB-2306; OriGene Technologies, Inc., Beijing, China) was used to detect the immunoactivity at room temperature for 2 h, followed by counterstaining with hematoxylin (OriGene Technologies, Inc.). The number of chondrocytes and positively stained ones were calculated by eye in the entire
articular cartilage using an optical microscope (version 510
UMA_cellSens19-Krishna-ch_00_01August 2013, Olympus
Corporation, Tokyo, Japan). The Osteoarthritis Research
Society International (OARSI)-Modified Mankin score
was calculated as previously described by Pritzker et al (21).

Statistical analysis. Data are presented as the mean ± standard
deviation. One-way analysis of variance followed by the
Least-Significant Difference post hoc test was used to deter-
mine if the difference among different groups was statistically
significant. SPSS version 22.0 (IBM Corporation, Armonk,
NY, USA) was used for data analysis. P<0.05 was considered
to indicate a statistically significant difference.

Results

Chondrogenic differentiation of ATDC5 cells. To examine the
chondrogenic differentiation of ATDC5 cells, expression of
Col II and Col X were analyzed by RT-qPCR. Expression of
Col II increased from day 1 and peaked at day 14, indicating
the early-stage differentiation of chondrocytes. Col X expres
sion gradually increased from day 7 to day 21, which indicated
hypertrophic and calcified chondrocytes (Fig. 1).

Toxicity of HF on chondrocytic ATDC5 cells. In order to
determine the appropriate dosage of HF for the subsequent
in vitro study, toxicity of HF on chondrocytic ATDC5 cells
was tested using CCK-8. Following incubation with HF of
different concentrations for 24, 48 and 72 h, a dose-dependent
and time-dependent decrease in cell viability was observed.
Cell viability was >84% after exposure to HF of 6.25, 12.5 and
25 ng/ml for 24 h. However, it was reduced to 63% following
treatment with 50 ng/ml HF and it was even lower in groups
affected with 100 and 200 ng/ml HF (Fig. 2A). From 24 to 72 h
of exposure, 6.25, 12.5 and 25 ng/ml HF had a negligible effect
on the cell viability. As for HF concentrations of 50, 100 and
200 ng/ml, the cell viability decreased in a time-dependent
manner (Fig. 2B). These findings suggested that HF of 6.25,
12.5 and 25 ng/ml did not affect chondrocytic ATDC5 cell
viability.

Halofuginone inhibited TGF-β1 signaling in chondrocytic
ATDC5 cells. To determine whether HF has a role in regu
lating TGF-β1 signaling in chondrocytic ATDC5 cells,
ATDC5 cells that underwent differentiation for 14 days were
used. Dose and time-dependent experiments were performed
followed by western blotting. The level of p-Smad2 protein
was reduced in a dose dependent manner, whereas the level
of total Smad2/3 remained unchanged (Fig. 3A). Next, the
present study determined the effect of HF pretreatment of
chondrocytic ATDC5 cells at three different time points
(4, 8 and 24 h) on the phosphorylation of Smad2 protein in
response to TGF-β1. The level of p-Smad2 was reduced with
time, whereas the level of total Smad2/3 remained unchanged
(Fig. 3B).

Halofuginone prevented articular cartilage degeneration by
inhibiting elevated TGF-β1 signaling. To investigate whether
HF can attenuate OA progression by way of oral gavage an
ACLT mouse model was established and HF was administered
every other day. Safranin O Fast Green staining was applied
to assess the severity of articular cartilage degeneration.
Deparaffinization of the slides was performed in xylene three
times for 5 min, followed by hydration in 100% alcohol twice
for 5 min, 95% alcohol for 5 min and 80% alcohol for 5 min.
Hematoxylin was added to slides for 1 min prior to hydrating
the slides gently in running water for 10 min. Slides were then
stained with 0.2% Fast Green for 5 min, and then subjected
to 1% acetic acid for 10 sec, 0.1% Safranin O for 2 min and
rinsing in water for 30 sec. Slides were hydrated in 95%
alkohol for 15 sec, 100% alcohol twice for 15 sec, followed
by 3 changes in xylene prior to cover-slipping the slides.
Proteoglycan loss in HF-treated group was reduced compared
with the vehicle-treated group (Fig. 4A). Furthermore, the
severity of articular cartilage degeneration was quantitatively
evaluated using OARSI-Modified Mankin score. HF-treated
group had a lower score compared with the vehicle-treated
group, indicating reduced articular cartilage damage (Fig. 4B).
Additionally, the expression of MMP-13 and Col X was lower in
the HF-treated group when compared with the vehicle-treated
group (Fig. 5A and B), indicating that HF had a protective role
in articular cartilage degeneration. The present study aimed
to investigate whether TGF-β1 had a role in the onset of OA. It was determined that the protein expression of TGF-β1 in articular cartilage was higher in the vehicle-treated group compared with the Sham group, and HF reduced its expression (Fig. 5C). Additionally, similar results were obtained for the expression of p-Smad2/3 in articular cartilage. HF-treated group had reduced expression of p-Smad2/3 compared with the vehicle-treated group (Fig. 5D). Altogether, these findings suggest HF attenuated articular cartilage degeneration by inhibiting elevated TGF-β1 signaling in articular cartilage.

**Discussion**

The present study transected the anterior cruciate ligament of C57BL/6 male mice to generate an unstable mechanical loading OA model. Compared with vehicle-treated group, HF administration attenuated OA progression by preserving articular cartilage. The underlying mechanisms of this phenomenon may be associated with its inhibitory effects on the elevated Smad2/3-dependent TGF-β1 pathway in articular cartilage during the onset of OA.
OA is a joint disorder characterized by articular cartilage degeneration, subchondral bone sclerosis, inflammation and osteophyte formation (22,23). Articular cartilage has a very limited reparative capacity, which makes it important to prevent cartilage damage at early stage. Articular cartilage is highly specialized connective tissue that transfers loads during weight bearing and joint motion (24,25). It primarily consists of extracellular matrix and chondrocytes. Chondrocytes are embedded in the extracellular matrix, producing a large number of collagenous extracellular matrix, including proteoglycan and Col II. Proteoglycan and Col II maintain the homeostasis and integrity of articular cartilage. During OA, the chondrocytes become hypertrophic and synthesize proinflammatory cytokines that contribute to their own destruction (26,27). Col X is a classic marker of hypertrophic differentiation of chondrocytes (28). MMP-13 is the primary collagenase synthesized by chondrocytes to damage aggrecan and Col II during OA. The OARSI-Modified Mankin scoring system is used to assess the severity of cartilage damage (21). The present study identified that HF treatment reduced the expression of Col X and MMP-13 in cartilage, lowered the OARSI score when compared with the vehicle-treated group. These findings demonstrated that HF improved the homeostasis and integrity of articular cartilage.

The role of TGF-β1 in articular cartilage is controversial. The reason for the conflicting role of TGF-β1 in articular cartilage remains to be elucidated. It has been previously reported that TGF-β1 is critical for the development of the knee joint. Serra et al. used transgenic mice where the type II TGF-β receptor was truncated (29). They identified the onset of early stage OA in the transgenic mice. At the age of 4 and 8 weeks, higher expression levels of Col X and lower levels of proteoglycan were detected in the transgenic mice when compared with their wild type littermates (29). Yang et al. noticed OA characteristics in Smad3-deficient mice, including loss of articular cartilage, osteophyte formation and elevated expression of Col X (30). These previous studies suggested that TGF-β1 is important for the chondrocyte hypertrophic differentiation in immature articular cartilage. Data from in vitro studies revealed that TGF-β1 may inhibit hypertrophic differentiation of chondrocytes through the regulation of articular cartilage matrix proteins and metalloproteases (31,32). These investigations support the view that TGF-β1 is a necessary protector against the onset of OA during the development of knee joints. Nevertheless, TGF-β1 may also contribute to the joint destruction. High level of TGF-β1 was detected in the articular cartilage of OA patients and mice OA models (33,34). The association between elevated TGF-β1 level and the onset of OA remains to be elucidated. The present study revealed that HF may inhibit Smad2/3-dependent TGF-β1 signaling in chondrocytes in vitro. In adult mice, compared with sham group, elevated levels of TGF-β1 and p-Smad2/3 were noticed in the vehicle-treated group and this was alleviated in the group treated with HF. The aforementioned findings suggested that HF prevented the cartilage damage during early-stage OA by inhibition of the elevated TGF-β1 signaling in articular cartilage.

HF has been used in a clinical trial for the treatment of chronic graft-versus host diseases and solid tumors, and had safe therapeutic effectiveness through oral application (35,36). The findings of the present study may add to its clinical application. To the best of our knowledge, the present study is the first to indicate that HF, by way of oral gavage, improved the homeostasis and integrity of articular cartilage. The underlying mechanism may be due to the
downregulation of abnormally elevated TGF-β1 signaling in the articular cartilage. These findings indicated that HF, administered by oral gavage and may be an effective strategy to prevent the onset of OA.

Acknowledgements

The present study was supported by grants from Major Science and Technology Projects in Xinjiang Uygur Autonomous Region (grant no. 201403123-3) and Joint Funds of the National Natural Science Foundation of China (grant no. U1503221).

References


