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Research paper

Novel chondrogenic and chondroprotective effects of the natural compound harmine

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ABSTRACT

A significant number of natural compounds have been shown to regulate the behavior of the cells, in collaboration with cellular proteins. CCN2/connective tissue growth factor (CTGF) has been reported to have essential roles in cartilage development, chondrocyte proliferation and differentiation as well as regulation of the extracellular matrix metabolism. Previous studies demonstrated the capability of CCN2 to regenerate surgical defects in articular cartilage of rat knee. Also, transgenic mice over-expressing cartilage-specific CCN2 were shown to be more resistant to aging-related cartilage degradation. We hypothesized that small molecules that induce CCN2 in chondrocytes could be novel candidates to increase the resistance to aging-related cartilage degradation, or even to correct cartilage degenerative changes incurred in OA. Therefore, this study screened a compound library and identified the β-carboline alkaloid harmine as a novel inducer of CCN2 in human chondrocytic HCS-2/8 cells and osteoarthritic articular chondrocytes. Harmine increased the expression of the cartilage markers aggrecan and *COL2α1*, as well as that of the master regulator of chondrogenesis, *SOX-9*. Moreover, harmine notably induced chondrogenesis of prechondrocytic ATDC5 cells in micromass cultures. The chondroprotective effect of harmine was investigated under inflammatory condition by stimulation with TNFα, and harmine was shown to ameliorate TNFα-induced decrease in expression of CCN2 and cartilage markers. These findings uncover novel chondrogenic effects of harmine and indicate harmine as a potential drug for prevention and/or repair of cartilage degradation.

1. Introduction

Articular cartilage is the typical permanent cartilage wherein chondrocytes do not undergo hypertrophic differentiation toward calcification, in contrast to those in the growth plate. Under healthy conditions, articular chondrocytes maintain the articular cartilage by balancing the synthesis of extracellular matrix (ECM) components and degradation by proteolytic enzymes, including matrix metalloproteinases (MMPs) and aggrecanases (e.g., ADAMTS4 and ADAMTS5) [1]. However, in degenerative joint diseases, such as osteoarthritis (OA), an imbalance between such anabolic and catabolic processes causes a continual degradation of the articular cartilage, which in severe cases, can be followed by subchondral bone exposure, osteophyte formation and eventual impaired joint mobility and pain. Several techniques and treatment modalities to repair cartilage or to prevent cartilage degradation have been described over the years, with great attention given to dietary supplements (e.g., chondroitin sulfate, hyaluronic acid) as well as to cell-based or growth factor-based regenerative therapeutics [2–7].

Previous studies have demonstrated that CCN family protein 2 (CCN2) or connective tissue growth factor (CTGF) promotes proliferation and maturation, but not hypertrophic differentiation of articular chondrocytes [8,9]. CCN2 has also been reported to regulate ECM metabolism, by interacting with a number of growth factors including bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β); and ECM components such as aggrecan, perlecan and fibronectin [10–13]. In osteoarthritic cartilage, overexpression of

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CCN2 is frequently observed among the clustered chondrocytes, which has been hypothesized to be a response to the injury, in attempt to regenerate the damaged cartilage by promoting the proliferation of and ECM synthesis by articular chondrocytes [14]. Indeed, Nishida et al. [7] demonstrated the capability of CCN2 to regenerate full-thickness articular defects and to ameliorate experimentally-induced OA in knee joints of rats. Furthermore, a recent study showed that cartilage-specific overexpression of *ccn2* resulted in the acquisition of an OA-resistant phenotype in mice [15].

Nevertheless, clinical application of growth factors encounters significant barriers related, for example, to its medical safety and cost. Therefore, we speculated that small molecules that induce CCN2 in chondrocytes could be novel candidates to increase the resistance to aging-related cartilage degradation, or even to correct cartilage degenerative changes incurred in OA. Based on these backgrounds, we herein screened an orphan ligand library and identified the β-carboline alkaloid harmine as an inducer of CCN2 in chondrocytes. Subsequent investigations uncovered novel properties of harmine that promotes chondrogenesis and protects cartilage against inflammatory damages.

2. Material and methods

2.1. Cell culture

A previously established human chondrocytic cell line (HCS-2/8 cells) [16] was cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 1% penicillin and streptomycin (Sigma, St Louis, MO, USA) and 1% L-glutamine (Invitrogen). HCS-2/8 cells were seeded at a density of 2×10^4, 2.5×10^5 or 5×10^5 in 96-well, 12-well or 6-well plate, respectively; and were incubated at 37 °C in 5% CO_2 for 24 h. Cells were then cultured under serum-starved condition (1% FBS) for another 24 h before stimulation with the compound(s), unless otherwise mentioned. Harmine and derivates (harmane, harmaline, harmane-1,2,3,4-tetrahydro-3-carboxylic acid) were purchased from Enzo Life Sciences (Tokyo, Japan).

Human articular chondrocytes (HACs) were isolated from cartilage samples obtained from femur or tibia of four patients with diagnosis of osteoarthritis undergoing total knee arthroplasty, under their informed consent. This study protocol was approved by the Ethical Committee for Human Research of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (#941). Briefly, cartilage samples were treated with 0.05% trypsin (Invitrogen) for 2 min, and 1 mm-thick samples were collected, minced and digested with 3 mg/mL collagenase-I (Worthington, Lakewood, NJ, USA) and 4 mg/mL dispase 2 (EIDIA, Tokyo, Japan) for 4 h at 37 °C in a water bath shaker. Chondrocytes were collected, filtered through a cell strainer (70 μm; BD Falcon; Bedford, MA, USA), and cultured in 10% FBS-containing DMEM low glucose (Invitrogen). Primary chondrocytes or the cells at passages 1 or 2 were seeded in the same conditions as those for HCS-2/8 cells and utilized in the experiments.

For analysis of harmine's effect under inflammatory condition, HCS-2/8 cells or HACs were stimulated with harmine with or without tumor necrosis factor alpha (TNFα; R&D systems, Minneapolis, MN, USA) and incubated for 24 h after serum starvation.

ATDC5 cells were maintained in DMEM and Ham's F-12 medium (DMEM/F-12, Invitrogen) containing 5% FBS. For micromass cultures, ATDC5 cells were suspended at a concentration of 2×10^7 per mL and seeded in micromasses of 10 μL in 48-well plates to simulate the high density of chondrogenic condensations [17]. One hour after plating, culture medium containing harmine (5 μM) or not was added to wells. Cells were cultured for 10 days and media were changed every other day.

2.2. Enzyme-linked immunosorbent assay (ELISA)-based screening of the small compound library

HCS-2/8 cells were used to screen an orphan ligand library (Enzo Life Sciences) that contained 84 compounds (10 μM, Table S1). In the first screening step, HCS-2/8 cells were incubated with them for 6 h, whereas in the second screening, cells were incubated with the selected compounds for 24 h. CCN2 protein levels in the supernatants were quantified by using a previously established sandwich ELISA system provided by Nichirei Corporation (Tokyo, Japan), as described previously [11,18].

2.3. Reverse transcription and real-time PCR analysis

Total cellular RNA was extracted by RNeasy (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Possible residual DNA was removed by DNase I digestion (QIAGEN RNase-Free DNase Set; QIAGEN). RNA samples were reverse-transcribed by using iScript cDNA synthesis kit (Bio-Rad; Hercules, CA, USA). Quantitative RT-PCR was performed to quantify the expression of the target gene by using iQ™ SYBR® Green supermix (Bio-Rad) and Chromo4™ real-time detector. The levels of mRNAs of interest were normalized to that of the reference gene S29. Primer sequences used herein are shown in Table 1.

2.4. Cell viability and toxicity assays

HCS-2/8 cells were incubated with different concentrations of harmine for 24 h. Cell viability was assessed by a colorimetric assay based on the bioreduction of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] by viable cells (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega, Madison, WI, USA), and cell toxicity was determined by a colorimetric assay of the lactate dehydrogenase (LDH) activity (Cytotoxicity detection kit; Roche Applied Sciences, Mannheim, Germany) in the supernatants, according to each manufacturer's instructions.

2.5. Western blot analysis

After 24-h incubation of HCS-2/8 cells with harmine, cells were washed with cold tris-buffer saline (TBS) and total cellular protein

Table 1
List of primer pairs used for real-time RT-PCR analysis.

Gene name (species)	Direction	Nucleotide sequence
S29 (human)	Sense	5′-TCTCGCTCTTGTCGTGTCTGTTC-3′
	Anti-sense	5′-ACACTGGCGGCACATATTGAGG-3′
CCN2 (human)	Sense	5′-TGCGAGGAGTGGGTGTGTGAC-3′
	Anti-sense	5′-TGGACCAGGCAGTTGGCTCTAATC-3′
Aggrecan (human)	Sense	5′-GGCATTTCAGCGGTTCCTTCTCC-3′
	Anti-sense	5′-CAGCAGTCGTCTCCTCTTCTACGG-3′
COL2α1	Sense	5′-TGGAGCAGCAAGAGCAAGGAGAA-3′
(human)	Anti-sense	5′-CCGTGGACAGCAGGCGTAGG-3′
SOX-9 (human)	Sense	5′-TGAAATCTGTTCTGGGAATGTT-3′
	Anti-sense	5′-ACTGCTGGTGTTCTGAGA-3′
S29 (mouse)	Sense	5′-CCACACGCCATCATCGGACTTC-3′
	Anti-sense	5′-GCAGAGGGTTCAGGGAGTAGGG-3′
CCN2 (mouse)	Sense	5′- ACACCGCACAGAACCACCACTC-3′
	Anti-sense	5′- GGCAGGCACAGGTCTTGATGAAC-3′
Aggrecan (mouse)	Sense	5′-ATGGCAACATTCACCTCTG-3′
	Anti-sense	5′-TAGCACTACCTCCGACATAG-3′
Col2α1	Sense	5′-GTCAATAATGGGAAGGCGGGAGG-3′
(mouse)	Anti-sense	5′-CGAGGGCAACAGCAGGTTCACATAC-3′