氏　　名	Hara, Emilio Satoshi
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学位論文題目	Novel chondrogenic and chondroprotective effects of the natural compound harmine. （軟骨分化促進，抗炎症作用を持つ天然物由来低分子化合物 Harmine）
学位論文審査委員	山本　敏男　教授　　窪木　拓男　教授 辻極　秀次　准教授

学位論文内容の要旨

I. Introduction

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. 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. In osteoarthritic cartilage, overexpression of 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. Indeed, Nishida et al. (2004) 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.

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.

II. Materials and methods

Three cell types were utilized in the experiments, namely, a human chondrocytic cell line HCS-2/8 cells, human articular chondrocytes and a mouse pre-chondrogenic cell line ATDC5. 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.

HCS-2/8 cells were used to screen an orphan ligand library (Enzo Life Sciences) for compounds that can induce CCN2 protein levels in the supernatants, which were quantified by using a previously established sandwich ELISA system provided by Nichirei Corporation (Tokyo, Japan).

Total cellular RNA was extracted by RNeasy, according to the manufacturer's instructions. RNA samples were reverse-transcribed and quantitated by real time RT-PCR for the expression of cartilage marker genes (aggrecan, col2a1, Sox-9 and CCN2). The levels of mRNAs of interest were normalized to that of the reference gene S29.

Cell viability was assessed by MTS assay, and cell toxicity was determined by a colorimetric assay of the lactate dehydrogenase (LDH) activity in the supernatants, according to each manufacturer's instructions.

For immunofluorescence studies against aggrecan, HCS-2/8 cells were incubated with different concentrations of harmine for 24 h. Detection of aggrecan (anti-human aggrecan antibody, R&D) was performed by an automated fluorescence imaging system (Cellomics ArrayScan™). Isotype control and the secondary antibody were mouse anti-human IgG (Pierce-Thermo Scientific) and Alexa488-conjugated goat anti-mouse IgG (Invitrogen) antibodies, respectively. Western blot was used to identify protein levels of Sox-9 upon stimulation with harmine.

For analysis of col2a1 promoter activity, ATDC5 cells were transfected with 1 mg of a luciferase reporter driven by the collagen type II a-1 (COL2a1) promoter enhancer and 0.1 mg of the control reporter plasmid encoding Renilla luciferase by electroporation. The COL2a1 reporter was a gift from Y. Yamada (NIDCR,NIH) and was made by replacing the lacZ gene in the original pKN185 [19] with a luciferase reporter gene, and is referred to as pKN185luc [20]. ATDC5 cells were then seeded onto 96-well plates and incubated with or without harmine (5 mM) for 72 h. The dual luciferase reporter assay system was used to determine firefly and constitutive Renilla luciferase expression. Relative luciferase activity (firefly luciferase reading/Renilla luciferase reading) was calculated for each well.

For micromass cultures, ATDC5 cells were suspended at a concentration of $2x10^7$ per mL and seeded in micromasses of 10 mL in 48-well plates to simulate the high density of chondrogenic condensations. One hour after plating, culture medium containing harmine (5 mM) or not was added to wells. Cells were cultured for 10 days and media were changed every other day. Ten-day micromass cultures of ATDC5 cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then incubated overnight with 0.1% alcian blue 8GX.

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 (TNFa; R&D systems, Minneapolis, MN, USA) and incubated for 24 h after serum starvation.

III. Results

Harmine increased the expression of the cartilage markers aggrecan and collagen type II (col2), as well as that of the master regulator of chondrogenesis, Sox-9. We then suspected a transcriptional induction of col2 by harmine, possibly through Sox-9. Thus, we examined the effect of harmine on the col2 transcription using a col2 luciferase reporter. The results of luciferase assay in a pre-chondrogenic cell line (ATDC5 cells) demonstrated that col2a1 promoter activity increased approximately by two folds upon harmine stimulation, compared to that of control sample (Fig. 4). In order to evaluate the chondrogenic effect of harmine, a micromass system using ATDC5 cells was carried out. The results showed that harmine notably induced chondrogenesis of ATDC5 cells in micromass cultures, with a great increase in the cartilage marker genes (aggrecan and col2) as well as CCN2. The chondroprotective effect of harmine was investigated under inflammatory condition by stimulation with tumor Snecrosis factor-alpha (TNFα), and harmine was shown to ameliorate TNFα-induced decrease in expression of CCN2 and cartilage marker genes.

IV. Conclusion

The results of our present study demonstrate the in vitro chondrogenic effect of harmine through increase in CCN2, SOX-9, aggrecan and COL2a1 levels, and suggest that harmine can be a useful compound for prevention and/or regeneration of cartilage degraded through aging or OA.

学位論文審査結果の要旨

結合組織成長因子（CCN2）は，軟骨細胞の増殖・成熟及び基質合成を促進し，軟骨特異的 CCN2 過剰発現マウスにおいては膝関節軟骨の加齢性変化に抵抗性を示すことや，実験的変形性関節症モデルの関節腔内に CCN2 を投与すると関節軟骨を修復できることが報告されており，CCN2 は変形性関節症の予防や治療に有効であると考えられている．そこで本研究では，変形性関節症の予防・治療を目的に，CCN2 の発現を亢進する新規化合物を網羅的に探索し，*in vitro* でその機能解析を行った．

スクリーニング実験では，ヒト軟骨肉腫由来軟骨細胞株（HCS-2/8）を 96-well プレートに播種し，86 種類のオーファンリガンド（Enzo Life Sciences）にて刺激し，培地中の CCN2 のタンパク量を ELISA 法にて定量した．同定した分子の細胞への毒性は，MTS 法(Promega)および LDH 毒性アッセイ(Roche)にて検討した．同定分子の軟骨分化に与える効果を検討するため，CCN2, Sox-9, Aggrecan, Type II Collagen, および MMP-1, 3, 13 の mRNA 量を real time RT-PCR 法にて定量した．炎症存在下における効果を評価するため，同定分子存在下で HCS-2/8 細胞を TNF-α で刺激し，同様の実験を行った．

スクリーニングの結果，検索したオーファンリガンドライブラリー中では harmine が CCN2 の発現を上昇させる唯一の分子として同定された．harmine は 5 μM の濃度において，細胞増殖を抑制するが，細胞毒性は認められず，軟骨細胞分化マーカーの遺伝子発現を促進し，軟骨細胞分化を促進した．また興味深いことに，harmine は TNF-α の刺激により誘導された MMP1,3,13 の遺伝子発現を抑制し，TNF-α の刺激により抑制された軟骨細胞分化を促進した．

本結果から，CCN2の発現を促進する因子として同定されたharmineは軟骨細胞分化促進作用，抗炎症作用の双方を持ち合わせる分子であり，植物成分の一つであり比較的安全で安価であるため，変形性関節症の新たな治療法につながる可能性が考えられた．

よって，審査委員会は本論文に博士（歯学）の学位論文としての価値を認める．