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Correlation between MMP-13 and HDAC7 expression in human knee osteoarthritis

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Abstract

Recent studies demonstrate that histone deacetylase (HDAC) inhibitors therapeutically prevent cartilage degradation in osteoarthritis (OA). Matrix metalloproteinase-13 (MMP-13) plays an important role in the pathogenesis of this disease and in the present study we investigated the correlation between HDACs and MMP-13. We found that HDAC inhibitor trichostatin A (TSA) could suppress both IL-1 dependent and independent MMP-13 mRNA expression (real time PCR) in human knee chondrocytes. Comparing the expression of different HDACs in cartilage from OA patients and healthy donors, HDAC7 showed a significant elevation in cartilage from OA patients. These results were confirmed by immunohistochemistry. Knockdown of HDAC7 by siRNA in SW 1353 human chondrosarcoma cells strongly suppressed IL-1 dependent induction of MMP-13 gene expression.

In conclusion, elevated HDAC7 expression in human OA may contribute to cartilage degradation via promoting MMP-13 gene expression and inhibition of HDACs by TSA or the selective inhibition of HDAC7 could be used therapeutically to stop OA progression.

Background

Osteoarthritis (OA) is a chronic degenerative joint disorder and a major cause of disability in the elderly. Characterized by progressive structural changes in articular cartilage, with persistent degeneration the disease eventually leads to loss of joint function. A significant feature of OA is the excessive production of inflammatory mediators [1-3], among which, pro-inflammatory cytokine interleukin-1 β (IL-1) plays a crucial role in the pathophysiology. IL-1 induces a cascade of inflammatory and catabolic events in chondrocytes, changing chondrocyte anabolism through suppression of proteoglycan and collagen synthesis and by enhancing matrix metalloproteinase (MMP) production.

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Several lines of evidence suggest that MMP-13 contributes to cartilage degradation in OA. MMP-13 expression is significantly higher in chondrocytes from cartilage of late stage OA compared to early OA or normal knee cartilage [4]. In explant cultures treated with a specific MMP-13 inhibitor, release of collagen degradation products from human OA cartilage is reduced [5]. Furthermore, transgenic mice over-expressing activated MMP-13 in the articular chondrocytes develop joint degradation similar to human OA [6]. Characterization of MMP-13 expression regulation in articular chondrocytes will contribute to understanding the molecular etiology of OA. Two families of HDACs have been identified: the classical HDAC family and the NAD⁺-dependent, so-called SIR2 family (sometimes called class III HDACs). Classical HDACs can be grouped into three classes (I, II, and IV) based on phylogeny [7]. Class I HDACs (HDAC 1, 2, 3, and 8) are related to yeast RPD3, and class II HDACs (HDAC 4, 5, 6, 7, 9, and 10) are more closely related to yeast HDA1 [8]. HDAC11 alone represents class IV, and HDAC11-related proteins have been described in all eukaryotic organisms with the exception of fungi [7]. Trichostatin A (TSA) is a HDAC inhibitor [8] with a broad spectrum of activity against class I and II HDACs, but not HDACs from the SIR2 family. Administration of these reagents to cells blocks histone deacetylation and leads to increased histone acetylation within gene expression in susceptible genes. There is also, however, many cases in which HDAC inhibitors act as repressors of gene expression [9-13].

Recently, HDACs have emerged as targets in cancer therapy and inflammatory diseases, including Rheumatoid Arthritis (RA) and OA [14-23], but it is still unclear which HDACs are specifically involved in cartilage degradation. These observations prompted us to investigate the HDAC expression in normal and OA cartilage and identify the specific HDAC that contributes to cartilage degradation in human OA.

Methods

Cartilage procurement and processing

All tissue samples were graded according to a modified Mankin scale [24], for which <3 points was normal and ≥5 points represented OA. Normal articular cartilage was harvested from femoral condyles and tibial plateaus of human tissue donors under approval by the Scripps Human Subjects Committee. Osteoarthritis cartilage was obtained from patients undergoing knee replacement surgery. Cartilage thickness ranged from 1.5 to 2.8 mm. Cartilage surfaces were rinsed with saline and parallel sections 5 mm apart were cut vertically from the cartilage surface onto subchondral bone with a scalpel. These cartilage strips were then resected from bone. Human chondrocytes were isolated and cultured as previously described [25]. Cartilage tissue was incubated with trypsin at 37°C for 10 minutes. Following removal of trypsin solution, tissue slices were treated for 12 to 16 hours with type IV clostridial collagenase in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal calf serum. After initial isolation, cells were kept in high-density cultures in DMEM (high glucose) supplemented with 10% CS, L-glutamine, and antibiotics and allowed to attach to the surface of the culture flasks. After cells had grown to confluence, they were split once (passage 1) and grown to confluence again in preparation for experiments [26].

Cell culture

Human knee chondrocytes were grown to confluence in 35 mm 6-well plates with 2 mL DMEM containing 10% FCS with or without Trichostatin A (TSA; SIGMA, Inc.) at 300 nM for 24h (Figure 4A). In parallel, cells were precultured 5h with 5 ng/mL IL-1 after which TSA was added at 300 nM and cultured additionally 24h (Figure 4B).

The knockdown experiments by small interference RNA (siRNA) was carried out on SW1353 human chondrosarcoma cells transfected with 25 nM siHDAC7 (Applied Biosystems, Inc.)

using Lipofectamine 2000 (Invitrogen Corporation) for 5h, following manufacturer's instructions.

Quantitative polymerase chain reaction

Total RNA was isolated from cartilage tissues or monolayer chondrocyte cultures using Trizol (Invitrogen, Inc., Carlsbad, CA, USA). Complementary DNA was produced using Ready-To-Go You Prime First Strand Beads (GE Health, Inc., USA) with 2 µg of total RNA and oligo (dT) primers. Messenger RNA expression of HDAC1-11 and MMP-13 was detected by real time RT-PCR with TaqMan Gene Expression Assay probe (Applied Biosystems, Inc.) using an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: 10 min at 95°C for initial denaturation, followed by 45 cycles at 95°C (15 s) and 60°C (1 min). The expression levels of HDACs and MMP-13 were defined from the threshold cycle (Ct) and relative values were calculated by the 2- $\Delta\Delta C_t$ method after normalizing expression to GAPDH.

Histology and immunohistochemistry

Cartilage tissues were fixed with 4% paraformaldehyde and stained with safranin O. HDAC7 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Paraffin-fixed samples were first deparaffinized in xylene substitute Pro-Par Clearant (Anatech Ltd., Battle Creek, MI, USA) and ethanol before rehydration in water. Following a wash with PBS, sections were blocked with 0.1% Tween20 with 3% normal goat serum for 30 min at room temperature. HDAC7 antibodies (1:50 dilution; 4 µg/mL) and normal rabbit IgG (4 µg/mL) as a negative control were applied and incubated overnight at 4°C. After washing with PBS, sections were incubated with biotinylated goat anti-mouse secondary antibody for 30 min (1:200; Vector Laboratories, Inc., Burlingame, CA, USA) and then incubated with Vectastain ABC-AP kit (AK-5000; Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. Finally, sections were stained with an alkaline phosphatase substrate kit (Vector Laboratories Inc., Burlingame, CA, USA).

Quantification and localization of signals throughout cartilage

To systematically assess HDAC7 localization throughout each cartilage zone, we counted positive and negative cells in a 50 × 50 µm grid (using a ×40 field objective) starting from the cartilage surface to the deep zone. This procedure was repeated a minimum of five times for each section. We based our identification of each zone on previously reported characteristics that comprise cell shape, morphology, orientation, and pericellular matrix (PM) deposition [27]. Thus, superficial zone (SZ) cells were characterized by their elongated shape, their parallel orientation relative to the surface, and their lack of extensive PM. These cells predominate within the first 50 µm. The middle zone (MZ) was distinguishable by the presence of rounded cells without an organized orientation relative to the surface, an ECM rich in proteoglycans, and evidence of PM. Conversely, deep zone (DZ) cells were recognized by extensive PM deposition and an organization of three or more cells in chondron groups arranged in columns. The depth of each zone was recorded for each section for comparative analysis on the frequency of positive signals in each zone. The frequency of positive cells was expressed as a percentage relative to the total number of cells counted in each zone.

Statistical analysis

Statistically significant differences between two groups were determined with t-tests. Results are presented as mean ± standard deviation. P values of less than 0.05 were considered statistically significant.

Results

Histone deacetylase inhibitor TSA modulate MMP-13 gene expression

When treating human knee chondrocytes with TSA only, the natural MMP-13 expression was suppressed to a small degree ($P=0.120$) and with IL-1 induction of MMP-13 the suppression was greater ($P=0.067$), but none of the results were statistically significant (Figure 1). Even though not significant, these results give a clue and further curiosity to which HDAC is affecting the MMP-13 expression.

HDAC7 expression is elevated in OA cartilage

HDAC 1-11 mRNA expression in human knee cartilage was determined by real time PCR. The expression of HDAC7 was significantly higher in OA than in normal cartilage (Figure 2A). Cartilage was obtained from 6 normal donors (age range: 19-49 years old; Mankin score: 0 to 2 points) and 10 OA donors (age range: 44-93 years old; Mankin score: 5 to 10 points) (Figure 2).

HDAC7 protein delocalized and elevated in OA cartilage

Normal and OA samples stained with Safranin O (Figures 2B and 2C) displayed different localization of HDAC7 positive cells. In normal cartilage, we detected positive cells in the middle zone (Figure 2D) whereas in OA cartilage, we detected many positive cells, especially in chondrocyte clusters (Figure 3D). Representative examples were a normal 48-year-old male (Mankin score: 1) and an 82-year-old male with OA (Mankin score: 9). Figure 2E presents the quantitative analysis of zonal distribution of HDAC7-expressing cells in 4 normal (range: 30 to 48 years old) and OA (range: 48 to 93 years old) donors. Complete erosion of the superficial zone was observed in OA cartilage. Moreover, significantly more positive cells were observed in middle and deep zones in OA cartilage, compared to that observed in normal middle and deep zones, respectively.

Knockdown of HDAC7 significantly decreases MMP-13 expression

To investigate the correlation between HDAC7 and MMP-13, HDAC7 was knocked down by siRNA in SW1353 human chondrosarcoma cells and mRNA expression of MMP-13 was measured by real time RT-PCR. HDAC7 knocked down cells were further stimulated with IL-1 and MMP-13 expression was measured again. Knocking down HDAC7 in SW1353 cells decreased both natural and IL-1-induced MMP-13 expression significantly, (Figure 3) strongly indicating interaction between HDAC7 and MMP-13.

Discussion

Onset and progression of OA is associated with changes in chondrocyte gene expression. HDACs balance histone acetyltransferases (HATs) and regulate gene transcription epigenetically, and thereby control the acetylation status of histone proteins and non-histone substrates. In general, acetylation of histones loosens nucleosomal structures, which promotes gene transcription. In contrast, deacetylation of histones stabilizes nucleosomal structures and represses gene transcription [28,29]. However, emerging evidence indicates that gene regulation by acetylation/deacetylation is more dynamic and complex, and that HATs also can act as repressors and HDACs as transcription activators. Indeed, global analysis of gene expression has shown that inhibition of HDAC activity results both in induction and repression of gene expression [30-35]. Recent studies demonstrated that HDAC inhibitors have therapeutic effects in cancer and inflammatory diseases [14-23]. Young *et al.* revealed that HDAC inhibitors modulate MMP gene expression in chondrocytes and block cartilage resorption [22], which was consistent with the present experimental results (Figure 1).

MMPs are a family of enzymes that collectively degrade components of the extracellular matrix (ECM). They are important in normal physiological processes such as development and wound healing where they appear in a low concentration. In contrast, aberrant MMP expression occurs in several disease states, including atherosclerosis, tumor invasion, and arthritic diseases [36, 37]. MMPs mediate irreversible matrix degradation and subsequent joint destruction in rheumatoid arthritis and OA. MMP-13 is expressed by chondrocytes and is critical for collagen degradation as it hydrolyzes type II collagen more efficiently than other collagenases [38]. Therefore, we wanted to see whether a specific HDAC is responsible for IL-1-induced MMP-13 expression and thus contributes to cartilage degradation in OA.

In the present study, we observed significant upregulation of HDAC7 in OA cartilage by real time RT-PCR and immunohistochemistry (Figure 3). HDAC7 is a member of class II HDACs, which comprises HDAC 4, 5, 6, 7, 9 and 10, all of which display cell type-restricted patterns of expression and contain a highly conserved C-terminal deacetylase catalytic domain. Class II HDACs also contain an N-terminal extension that links them to specific transcription factors and confers responsiveness to a variety of signal transduction pathways serving as a link between the genome and the extracellular environment [39]. Disruption of the HDAC7 gene in mice results in embryonic lethality due to a failure of endothelial cell-cell adhesion and consequent dilatation and rupture of blood vessels. HDAC7 represses MMP-10 gene transcription by associating with myocyte enhancer factor-2 (MEF2), a direct activator of MMP-10 transcription and an essential regulator of angiogenesis [40]. Recently, Jensen *et al.* demonstrated that HDAC7 associates with Runx2 and represses its activity during osteoblast maturation [41]. Runx2 is required for MMP-13 promoter activity induced by IL-1 [42]. Increased expression of Runx2 in OA cartilage may contribute to increased expression of MMP-13 [43]. Kawaguchi *et al.* proposed that endochondral ossification signals, in which Runx2 plays a central role, may be important for OA progression [44,45]. Thus, we first hypothesized that HDAC7 may repress Runx2 activity and contribute to blockade of IL-1 induction of MMP-13 in articular chondrocytes. In our study, however, knockdown of HDAC7 by RNA interference resulted in the opposite effect (Figure 4), therefore suggesting that HDAC7 may activate IL-1 induction of MMP-13 in chondrocytes. Given the number of other mechanisms for regulation of MMP-13 expression [46-52], it is likely that HDAC7 interacts with many other factors. The human genome contains only 18 HDAC genes, but more than 1,800 genes are predicted to encode transcription factors [53]. Thus, DNA binding proteins dictate specificity, while HDACs and other co-factors serve as non-specific, broadly acting modulators of gene expression, even though class II HDACs have cell type-restricted specificity.

Certain HDAC inhibitors may selectively inhibit different HDACs [54-58]. Recently, Schuetz *et al.* characterized the structure and enzymatic activity of the catalytic domain of human HDAC7 [59], making development of a modulator specific for HDAC7 is possible in the near future. Given the decrease in chondrocyte MMP-13 expression following HDAC7 suppression observed in our study (Figure 4), a specific HDAC7 inhibitor may be an effective therapeutic agent for human knee OA.

Conclusions

Our findings support the idea that HDAC7 expression is elevated in human OA cartilage and promotes IL-1 induction of MMP13, contributing to cartilage degradation. Many enigmatic interactions remain unclear, and further studies are needed to elucidate the mechanism of cartilage degradation by MMP-13 in OA.

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List of abbreviations

MMP	Matrix metalloproteinase
OA	osteoarthritis
HDAC	histone deacetylase
RT-PCR	real-time reverse transcriptase-polymerase chain reaction
IL-1	interleukin-1 β
TSA	trichostatin A

Figure 1. TSA suppresses both natural and IL-1-induced MMP-13 expression

TSA lowered both natural and IL-1 induced MMP-13 expression, although the effect was not statistically significant. Real time PCR results from (A) Human knee chondrocytes (n=6, age range: 19-66 years old) treated or untreated with 300 nM of TSA for 24 h. (B) chondrocytes stimulated with IL-1 (5 ng/mL) 5 h and then treated or untreated with TSA (300 nM) 24h. GAPDH gene expression was used for normalization. Results are expressed as –fold changes relative to a value of one for untreated control cells. $P=0.120$ (A). Results are expressed as –fold changes relative to a value of one for untreated control cells after 5 h of IL-1 stimulation. $P=0.067$ (B)

Figure 2. Localization of HDAC7 in normal and OA knee cartilage

A. HDAC 1-11 mRNA expression in human knee cartilage was determined by real time RT-PCR. In normal samples, HDAC3 was the most abundantly expressed HDAC (Figure 1).

HDAC7 had significantly higher expression in OA than in normal cartilage. Cartilage was obtained from 6 normal donors (mean age: 30.8 years; range: 19 to 49 years; Mankin score: 0 to 2 points) and 10 OA donors (mean age: 71.6 years; range: 44-93 years old; Mankin score: 5 to 10 points) (Figure 1). The results are expressed as mean $\pm$ SD. * P <0.05 B-F.

HDAC7 localization was examined in tissue from 4 normal donors (age range: 30-48 years old) and 4 OA donors (age range: 48-93 years old). HDAC7-positive cells were more frequent in OA cartilage than in normal cartilage. In OA cartilage, nearly all cells within chondrocyte clusters stained for HDAC7. In normal cartilage, positive cells were more frequent in middle and deep zones. The immuno-reactive product is dark red. Magnifications: $\times 10$ (a,b,c,d). The number of HDAC7 positive cells was counted in the superficial, middle, and deep zones of sections from normal (n=4) and OA (n=4) cartilage with specific antibodies. In normal cartilage, percentage of HDAC7-positive cells was highest in the middle zone. The superficial zone in OA cartilage was eroded. The OA middle and deep zones had significantly more HDAC7-positive cells than normal middle and deep zones, respectively. The results are expressed as mean $\pm$ SD. * P <0.05, ** P <0.01

Figure 3. Knockdown of HDAC7 by siRNA in SW1353 human chondrosarcoma cells

Real time PCR results of MMP-13 expression in HDAC7 knocked down cells with and without the additional 5h culturing in IL-1 (5 ng/mL). Knockdown of HDAC7 decreased both natural and IL-1 induced MMP-13 expression.

Data represent mean $\pm$ SD (n=2, in duplicate). ***P<0.001