Effects of Trichostatin A on the Chondrogenesis from Human Mesenchymal Stem Cells

Jimin Lee¹ · Gun-Il Im¹

Received: 27 July 2016/Revised: 17 August 2016/Accepted: 12 September 2016/Published online: 7 March 2017
© The Korean Tissue Engineering and Regenerative Medicine Society and Springer Science+Business Media Dordrecht 2017

Abstract Histone deacetylase inhibitors (HDACi) are a class of compounds that suppress the function of histone deacetylases (HDACs). This study was performed to examine the effects of Trichostatin A (TSA), a typical HDACi, on chondrogenesis of human bone marrow mesenchymal stem cells (hBMMSCs) and related molecular pathways. After evaluating the concentration for cytotoxicity and HDAC activity, hBMMSCs underwent chondrogenic differentiation in pellet culture with or without TSA for 21 days. The weight of TSA-treated pellets was 25% lower than that of untreated pellets. DNA level was not significantly different, but glycosaminoglycan content per DNA level was lower in TSA-treated pellets than that of untreated pellets. Gene expression of the chondrogenic markers (SOX9, Aggrecan, and Col2A1) decreased by 12.9-fold, 8.9-fold, and 7.6-fold respectively in TSA-treated pellets compared with that in TSA-untreated pellets. TSA-treated pellets had lower cell density and lower proteoglycan staining content compared with those of TSA-untreated pellets. A microarray analysis from TSA-treated pellets showed that 1,467 chondrogenic-related genes were downregulated and 1,524 were upregulated by more than 2-fold compared with TSA-untreated pellets. Col10A1, TGF- β 3, and SOX9 decreased significantly by 10-fold, 2.1-fold, and 3.2-fold respectively in TSA-treated pellets compared with those in untreated pellets, whereas expression of BMP4 and FGFR3 increased significantly by 2.1-fold and 5.4-fold respectively. It is concluded that TSA inhibits chondrogenesis and does not seem to be useful for cartilage tissue engineering of hBMMSCs.

Keywords HDAC inhibitor · TSA · Chondrogenesis · Differentiation · Gene profiling

1 Introduction

Articular cartilage has limited capacity for self-repair. Damage to articular cartilage usually leads to osteoarthritis. Recently, cell therapy has been applied to treat hitherto intractable defects in articular cartilage. Of the cell sources,

mesenchymal stem cells or mesenchymal stromal cells (MSCs) has been a promising tool for clinical applications in cartilage regeneration [1]. hMSCs (human mesenchymal stem cells) have been extensively studied for chondrogenesis *in vitro* [2]. Considering that expensive peptide growth factors are necessary for the induction of chondrogenesis from hMSCs, identification of small molecules that can modify chondrogenesis of hMSC may facilitate the potential application of hMSCs for the cartilage repair [3].

Histone deacetylases (HDAC) are enzymes that silence transcription by modifying chromatin folding. HDAC catalyze the transfer of acetyl groups from acetyl coenzyme A to the ϵ -amino groups of lysine residues in the histone tail, which leads to chromatin condensation, reducing the accessibility of DNA-binding transcription factors [4]. Histone acetylation is closely related to other epigenetic

Electronic supplementary material The online version of this article (doi:10.1007/s13770-017-0041-6) contains supplementary material, which is available to authorized users.

✉ Gun-Il Im
gunil@duih.org
Jimin Lee
jimin15@dongguk.edu

¹ Department of Orthopaedics, Dongguk University Ilsan Hospital, 27, Dongguk-ro, Goyang 10326, Republic of Korea

regulation including DNA and histone methylation [2, 5]. Inhibition of HDACs decreases stem cells self-renewal by enhancing expression of differentiation markers [6] and accelerating induced differentiation into osteoblasts [7].

Trichostatin A (TSA) belongs to one of the four classes of histone deacetylase inhibitors (HDACi) and has been used as a therapeutic agent for various cancers [8–10]. TSA also inhibit the expression of destructive metalloproteinases in chondrocytes, which may make it useful as a chondroprotective agent to treat osteoarthritis [11]. Varying effects of TSA on chondrogenesis were reported. El-Serafi et al. found that TSA enhanced cartilage matrix formation and chondrogenic structure in hBMMSC pellets cultured in chondrogenic media [12]. On the other hand, Wang et al. reported that TSA significantly decreased chondrogenic gene expression and the synthesis of chondrogenic proteins in a dose-dependent manner during transforming growth factor (TGF)- β 1-induced chondrogenesis by inhibiting of TGF- β 1-induced Sp1 expression [3]. To explore the utility of TSA for cartilage tissue engineering, it is necessary to reconfirm the effect of this HDACi on chondrogenesis and the mechanisms underlying the effect. Therefore we investigated the effect of TSA on the chondrogenic differentiation of MSCs and the molecular mechanisms related to TSA treatment. Finally, we analyzed gene expression patterns by TSA in chondrogenesis.

2 Materials and methods

2.1 Cell preparation

Human bone marrow mesenchymal stem cells (hBMMSCs) were purchased from Lonza (Allendale, NJ, USA) and maintained in Minimum Essential Medium Alpha Modification Medium (Welgene Inc., Daegu, Republic of Korea) supplemented with penicillin–streptomycin 100 X (Welgene), and 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). Cells were grown in a CO₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 5% CO₂ atmosphere and 37°C.

Cytotoxicity of TSA (Sigma, St. Louis, MO, USA) on hBMMSCs was measured with the Cell Counting Kit-8 (DOJINDO, ENZO Life Science, Kumamoto, Japan) to define the half maximal inhibitory concentration (IC₅₀) of TSA influencing growth of hBMMSCs (3.3 μ M, Fig. 1B).

2.2 HDAC inhibitor activity assay

Cells were seeded at the density of 1×10^5 cells in 60 mm dish and were treated with TSA at 0, 0.5, and 5 μ M for 72 hrs. The cells were harvested using Histone Deacetylase Assay Kit, Fluorometric (Sigma) and then were analyzed by Microplate spectrophotometer (SpectraMax M3,

Molecular Devices, Sunnyvale, CA, USA) with 562 nm laser excitation beam (Fig. 1C).

HDAC activity was calculated as follows: [HDAC activity] = [Enzyme sample assay] – [No enzyme control assay]. The efficacy of inhibition of the test compounds on the HDAC activity was calculated as follows: [inhibition effect] = [Test compound assay – No enzyme control] – [HDAC activity].

2.3 Chondrogenic induction of hBMMSCs and TSA treatment

At confluence hBMMSCs were trypsinized and aliquots of 2.5×10^5 cells were spun down at 500 g for 10 min and were cultured as pellets. The cells were induced to differentiate in a chondrogenic medium consisting of Dulbecco's modified Eagle's medium/F-12 1:1 Nutrient Mixture (DMEM/F-12) (Welgene) supplemented with 1% v/v 100 $\times$ Insulin-Transferrin-Selenium (Gibco), 75 mg/mL bovine serum albumin (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 M L-ascorbate (Sigma), 0.1 M L-proline (Sigma), dexamethasone 10^{-7} M (Sigma), and 10 μ g/mL recombinant human TGF- β 3 (R&D Systems, Minneapolis, MN, USA) and the absence or presence of TSA (3.3 μ M). The media was changed every other day, and the pellets were harvested on day 21. The pellets were washed twice in DPBS and fixed in 4% paraformaldehyde for 1 h. The size (means diameter) and wet weight of the pellets were measured and used for further analysis.

2.4 DNA and RNA extraction

Genomic DNA (gDNA) from each pellet for day 21 was prepared using a GeneAll Tissue SV mini Kit (GeneAll, Seoul, Korea) in accordance with the manufacturer's instructions. DNA content was determined by a nanophotometer (Implen, Inc., Westlake Village, CA, USA). Total RNA was extracted on day 14 by TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). complementary DNA (cDNA) was synthesized from total RNA using the Maxime RT oligo(dT) preMix kit (iNtRON Biotechnology, Seongnam-si, Korea) according to the manufacturer's instructions. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed on the Rotor-Gene Q 5 plex PCR cycler (Qiagen, Hilden, Germany). All samples were evaluated in triplicate.

2.5 Glycosaminoglycan contents and histology

Glycosaminoglycan (GAG) content was determined with a Blyscan kit (Biocolor life science, Carrickfergus, UK) in accordance with the manufacturer's protocol. GAG content is described as GAG (μ g) per DNA (μ g).

Fig. 1 Cytotoxicity and suppression of HDAC by TSA. **A** Chemical structure of TSA, **B** The cytotoxicity of TSA to hBMMSCs was measured by the CCK-8 assay on day 1 and 3. Values are presented as percentage to those of vehicle. **C** HDAC activity assay was performed

After inducing chondrogenesis for 21 days, the pellets were harvested and analyzed. The fixed pellets were prepared for paraffin embedding, and 4 μm thick sections were subjected to Safranin-O, Alcian blue, and hematoxylin and eosin (H&E) staining to verify chondrogenic differentiation of hBMMSCs.

2.6 Gene expression profiling with oligonucleotide microarray

After the fragmented cRNA was prepared, it was resuspended in $2 \times$ hybridization buffer and pipetted onto the assembled Agilent Human Whole Genome 44 K (V2) microarray. The arrays were hybridized for 17 h at 65°C using the Agilent Hybridization oven (Agilent Technologies, Santa Clara, CA, USA). The hybridized microarrays were washed using the manufacturer's washing protocol (Agilent Technologies).

The microarray images were scanned with the Agilent DNA microarray Scanner, and the data were quantified using Agilent Feature Extraction software 9.3.2.1. All data

in hBMMSCs on day 3 after TSA treatment. Data are mean $\pm$ standard deviation of three experiments. $*p < 0.05$, HDAC Histone deacetylase, TSA Trichostatin, hBMMSCs human bone marrow mesenchymal stem cells, CCK-8 Cell Counting Kit-8

were normalized using the intensity-dependent normalization method (locally weighted scatterplot smoothing). The averages of the normalized ratios were calculated by dividing the average of the normalized signal channel intensity by the average of the normalized control channel intensity. GeneSpringGX 7.3.1 (Agilent Technologies) was used to select the genes that showed more than two-fold differences in expression.

2.7 Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis

We selected three chondrogenic markers (SOX9, Aggrecan, and Col2A1) to evaluate chondrogenesis. We selected upregulated BMP4 and FGFR3 and downregulated genes (Col10A1, SOX9 and TGF- β 3) by TSA treatment from the microarray results in order to confirm gene expression profiling. The qRT-PCR reaction conditions were as follows: activation for 10 min at 95°C and denaturation 45 cycles for 5 s at 95°C on each cycle, annealing for 10 s at 60°C, and extension for 15 s at 72°C. For PCR

Fig. 2 TSA inhibits chondrogenic differentiation of hBMMSCs. **A** Representative images of chondrogenic pellets after day 21 (scale = 1 mm), DNA quantity, and GAG per DNA. **B** qRT-PCR of chondrogenic markers in the chondrogenic pellets. **C** Hematoxylin

and eosin (H&E), Alcian blue, and Safranin-O staining of chondrogenic pellets (scale bars = 0.3 mm). $n = 3$, $*p < 0.05$, NS no significant, GAG Glycosaminoglycan

amplification, *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) was used as an internal control and the primers are provided in supplemental Table S1. The cycle threshold (Ct) value of each gene was estimated for each reverse transcript sample. All executed experiments were performed in triplicate.

2.8 Statistical analysis

All data were expressed as mean $\pm$ standard deviation. Statistical comparisons were made using the Mann–Whitney *U*-test and SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). The Statistical significant *p* values were set at $p < 0.05$.

3 Results

3.1 Cell viability and HDAC inhibition by TSA

Cytotoxicity of TSA was measured for 24 h and 72 h. At 24 h, cytotoxicity of TSA was definite from the

concentration of 50 μ M. The IC_{50} (72 h) value of TSA after treating the hBMMSCs for 72 h was 3.3 μ M (Fig. 1B). HDAC activity was suppressed by 26% at 0.5 μ M and by 41% at 5 μ M (Fig. 1C). This concentration of TSA (3.3 μ M) was used in the hBMMSC chondrogenic pellet culture.

3.2 TSA inhibits chondrogenic differentiation of hBMMSCs

The pellets treated with TSA had 1.2-fold diameters ($p < 0.05$) but the wet weights of the TSA-treated pellets were 25% lower than those of the untreated pellets ($p < 0.05$). GAG per DNA level was also 25% lower in TSA-treated pellets than that in TSA-untreated pellets ($p < 0.05$), on the other hand, DNA level was no significant difference between TSA-treated pellets and TSA-untreated pellets (Fig. 2A). Gene expression of the chondrogenic markers SOX9, Aggrecan, and Col2A1 decreased significantly by 12.9-fold, 8.9-fold, and 7.6-fold in TSA-treated pellets compared with that in TSA-untreated pellets ($p < 0.05$) (Fig. 2B). The H&E, Safranin-O,

Fig. 3 Gene expression profiling and hierarchical clustering of TSA-untreated and TSA-treated hBMMSCs. TSA-treated groups were treated with 3.3 μ M TSA for 14 days. Twenty-one genes were related

with chondrogenesis and showed more than a two-fold expression difference. *Red* denotes over expression and *green* denotes under expression in response to TSA treatment

and Alcian blue staining demonstrated that TSA-treated pellets had lower cell density and lower proteoglycan staining intensity than those of TSA-untreated pellets (Fig. 2C). Overall, these findings suggest that TSA inhibits chondrogenesis of hBMMSCs.

3.3 Microarray reveals upregulated and downregulated gene lists in response to TSA treatment

We performed microarray with mRNA isolated from chondrogenic pellets on day 21. The heat maps, the result of microarray, were subject to a hierarchical clustering analysis. The microarray analysis showed that 1,467 chondrogenic-related genes were downregulated and 1,524 were upregulated by more than two-fold expression differences. Of them, 21 genes have reported to be more closely associated with chondrogenesis (Fig. 3 and Table 1). Of the 21 genes, Col10A1 was downregulated more than 10-fold, TGF- β 3 was 2.1-fold downregulated and SOX9 was 3.2-fold downregulated. BMP4 and FGFR3 were upregulated 2.1-fold and 5.4-fold respectively. These five genes (Col10A1, TGF- β 3, SOX9, BMP4, and FGFR3) were chosen for the qRT-PCR analysis.

3.4 qRT-PCR confirms downregulation of Col10A1, TGF- β 3, and SOX9 and upregulation of BMP4 and FGFR3 by TSA treatment

The microarray results were further confirmed by qRT-PCR of selected genes. Col10A1, TGF- β 3 and SOX9 were decreased significantly by 3.3-fold, 9.6-fold and 11-fold respectively in TSA-treated pellets compared with untreated pellets ($p < 0.05$) (Fig. 4A), whereas BMP4 and FGFR3 expression increased significantly by 1.6-fold and 3.3-fold respectively ($p < 0.05$) (Fig. 4B). These mean that TSA caused suppression of TGF- β 3 and SOX9, which are key mediators of chondrogenesis, and induction of BMP4 and FGFR3, which promotes osteogenesis.

4 Discussion

The results show that TSA inhibits chondrogenesis of hBMMSCs, which agrees with those of Wang et al. who showed that TSA has suppressive effect on chondrogenesis rather than enhancing effect. In this sense, TSA is detrimental rather than contributory to cartilage tissue engineering. TSA suppressed expression of SOX9, the key chondrogenic transcription factor, and Col2A1 and

Table 1 Summary of changes in gene expression (more than 2-fold) for the core set of TSA regulated genes in hBMMSCs

Accession#	Gene symbol	Gene name	Reported function
Repressed			
NM_000493	Col10A1	Collagen, type X, alpha 1	Chondrocyte hypertrophy
NM_006208	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	Mutation results in vascular calcification
NM_002607	PDGFA	Platelet-derived growth factor alpha polypeptide	Mitogen and chemotactic factor for mesenchymal cells
NM_004370	Col12A1	Collagen, type XII, alpha 1	Crosslinking of collagen type I
NM_001945	HBEGF	Heparin-binding EGF-like growth factor	Mitogenic and chemotactic factor
NM_000346	SOX9	SRY (sex determining region Y)-box 9	Key chondrogenic transcription factor
NM_015170	SULF1	Sulfatase 1	Modulates the effects of heparan sulfate Needed for normal chondrogenesis
NM_001901	CTGF	Connective tissue growth factor	Important roles in skeletal development, and tissue wound repair
NM_006902	PRRX1	Paired related homeobox 1	Transcription coactivator, regulate differentiation of mesenchymal precursors.
NM_008711	NOG	Noggin	BMP antagonist
NM_003239	TGF- β 3	Transforming Growth Factor, beta 3	Important chondrogenic growth factor
NM_001554	CYR61	Cysteine-rich, angiogenic inducer, 61	Regulates cell adhesion, migration, proliferation, differentiation
Induced			
NM_003068	SNAI2	Snail family zinc finger 2	Involved in epithelial-mesenchymal transitions
NM_001202	BMP4	Bone morphogenetic protein 4	Important role in the onset of endochondral bone formation
NM_002129	HMGB2	High mobility group box 2	Negatively regulate RUNX2 in MSC chondrocyte differentiation
NM_001463	FRZB	Frizzled-related protein	Wnt-binding protein important in embryonic development
NM_000557	GDF5	Growth differentiation factor 5	Enhances <i>in vitro</i> MSC chondrogenesis and hypertrophy
NM_019886	CHST7	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	Transfer sulfate to carbohydrate groups in glycoproteins and glycolipids
NM_003278	CLEC3B	C-type lectin domain family 3, member B	Bone matrix protein expressed coincidentally with mineralization
NM_001083602	PTCH1	Patched 1	Receptor for sonic hedgehog, a secreted molecule important in embryogenesis
NM_000142	FGFR3	Fibroblast growth factor receptor 3	Receptor for FGF, regulated chondrocyte proliferation

Aggrecan, two major cartilage matrix components, as well as decreasing total proteoglycan content in the pellet. These results suggest that TSA is not useful for cartilage tissue engineering of hBMMSCs.

One of the key differences between this study and previous studies was the concentration of TSA (3.3 μ M) used to treat the hBMMSCs, which was much higher than those ($\sim$ 100 nM) used in previous studies. The high concentration, which was considered cytotoxic, was still used in this study because HDAC was substantially inhibited on that concentration in hBMMSCs. TSA treatment was done in a three-dimensional (3-D) chondrogenic pellet culture model. This model provides different environment from monolayer culture in which most HDACi-related

experiments have been performed. A 3-D culture is a standard and necessary condition for chondrogenic differentiation of hBMMSCs. With the pellet culture, we measured the diameter of pellets, and the TSA-treated pellets had greater diameter than TSA-untreated pellets. According to Jahn et al., the formation of a surface fibrous-tissue-like cell layer surrounding the inner cells of the pellets was detected by day 19, and the pellets on day 19 were much smaller than day 7 [13].

No difference in DNA level was detected between TSA-treated and untreated pellets in the current study, indicating that cell proliferation was not affected by TSA in the chondrogenic pellet culture environment. While the concentration used in the current study not only effectively

Fig. 4 qRT-PCR validation of TSA-induced gene expression changes. **A** Col10A1, TGF-β3, and SOX9 were downregulated, and **B** BMP4 and FGFR3 were upregulated in TSA-treated pellets; $n = 3$, $*p < 0.05$

suppressed HDAC activity but also strongly inhibited cell proliferation in monolayer culture, hBMMSCs were more resistant to the toxicity of TSA in the chondrogenic pellet culture. The relatively hypoxic conditions inside the pellets or components of the chondrogenic medium including TGF-β may have moderated the toxic effect of TSA. Also, activation of proliferative genes, such as BMP4 and FGFR3, may have mitigated the inhibitory effect of TSA on cell proliferation.

The microarray results provide a glimpse into the molecular pathways involved in TSA-mediated suppression of chondrogenesis. Although not applicable to all listed genes and genes have several different functions, the suppressed genes which are related to chondrogenic differentiation include SOX9, TGF-β3, SULF1, CTGF, Prrx1. On the other hand, FGFR3, which is related to chondrocyte proliferation, or factors related to osteogenic differentiation, such as BMP4, FRZB, GDF5, and CLEC3B increased. These results suggest that suppression of HDAC by TSA have differential effects on the expression of genes involved in the proliferation and differentiation of hBMMSCs.

The limitation of this study is that only *in vitro* data was presented to show that TSA inhibits chondrogenesis.

Animal models that can test cartilage regeneration or improvement of osteoarthritis could have strengthened the result of *in vitro* investigations. Also, an *in vitro* design that enhances the action of HDAC could have produced interesting results. Still, the findings from this study provide a corroboratory data on the concept that TSA is not useful for cartilage regeneration. In conclusion, TSA inhibits chondrogenesis and is not useful for enhancing cartilage tissue engineering of hBMMSCs.

Acknowledgements This study was financially supported by the National Research Foundation (NRF) funded by the Korean government (2015R1A2A1A09002793).

Compliance with ethical standards

Conflicts of interest The authors have declared that there is no conflict of interest.

Ethical statement There are no animal experiments carried out for this article.

References

1. Tuan RS, Boland G, Tuli R. Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther*. 2003;5:32–45.
2. Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. *Blood*. 2003;102:3483–93.
3. Wang JP, Wen MH, Chen YT, Lee HH, Chiang ER, Lee YT, et al. Trichostatin A inhibits TGF-β1 induced *in vitro* chondrogenesis of hMSCs through Sp1 suppression. *Differentiation*. 2011;81:119–26.
4. Sengupta N, Seto E. Regulation of histone deacetylase activities. *J Cell Biochem*. 2004;93:57–67.
5. Aoyama T, Okamoto T, Kohno Y, Fukiage K, Otsuka S, Furu M, et al. Cell-specific epigenetic regulation of ChM-I gene expression: crosstalk between DNA methylation and histone acetylation. *Biochem Biophys Res Commun*. 2008;365:124–30.
6. Karantzi E, Schulz H, Hummel O, Hubner N, Hatzopoulos A, Kretsovali A. Histone deacetylase inhibition accelerates the early events of stem cell differentiation: transcriptomic and epigenetic analysis. *Genome Biol*. 2008;9:R65.
7. Chen TH, Chen WM, Hsu KH, Kuo CD, Hung SC. Sodium butyrate activates ERK to regulate differentiation of mesenchymal stem cells. *Biochem Biophys Res Commun*. 2007;355:913–8.
8. Katsura T, Iwai S, Ota Y, Shimizu H, Ikuta K, Yura Y. The effects of trichostatin A on the oncolytic ability of herpes simplex virus for oral squamous cell carcinoma cells. *Cancer Gene Ther*. 2009;16:237–45.
9. Vigushin DM, Ali S, Pace PE, Mirsaidi N, Ito K, Adcock I, et al. Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer *in vivo*. *Clin Cancer Res*. 2001;7:971–6.
10. Zhang S, Cai X, Huang F, Zhong W, Yu Z. Effect of trichostatin A on viability and microRNA expression in human pancreatic cancer cell line BxPC-3. *Exp Oncol*. 2008;30:265–8.
11. Young DA, Lakey RL, Pennington CJ, Jones D, Kevorkian L, Edwards DR, et al. Histone deacetylase inhibitors modulate metalloproteinase gene expression in chondrocytes and block cartilage resorption. *Arthritis Res Ther*. 2005;7:R503–12.

12. El-Serafi AT, Oreffo RO, Roach HI. Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: differential effects of 5-aza-deoxycytidine and trichostatin A. *Differentiation*. 2011;81:35–41.
13. Jähn K, Richards RG, Archer CW, Stoddart MJ. Pellet culture model for human primary osteoblasts. *Eur Cells Mater*. 2010;20:149–61.