In Vitro Anti-Inflammation and Chondrogenic Differentiation Effects of Inclusion Nanocomplexes of Hyaluronic Acid-Beta Cyclodextrin and Simvastatin

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Abstract The aim of this study was to prepare inclusion nanocomplexes of hyaluronic acid- β -cyclodextrin and simvastatin (HA- β -CD/SIM) and evaluate *in vitro* anti-inflammation effects on lipopolysaccharide (LPS)-activated synoviocytes and chondrogenic differentiation effects on rat adipose-derived stem cells (rADSCs). The β -CD moieties in HA- β -CD could incorporate SIM to form HA- β -CD/SIM nanocomplexes with diameters of 297–350 nm. HA- β -CD/SIM resulted in long-term release of SIM from the nanocomplexes for up to 63 days in a sustained manner. *In vitro* studies revealed that HA- β -CD/SIM nanocomplexes were able to effectively and dose-dependently suppress the mRNA expression levels of pro-inflammatory markers such as matrix metalloproteinase-3 (MMP-3), MMP-13, cyclooxygenase-2 (COX-2), a disintegrin and metalloproteinase with thrombospondin motifs-5 (ADAMTS-5), interleukin-6 (IL-6), and tumor necrosis factor (TNF- α) in LPS-stimulated synoviocytes. HA- β -CD/SIM-treated rADSCs significantly and dose-dependently enhanced mRNA expressions of aggrecan, collagen type II (COL2A1), and collagen type X (COL10A1), implying that HA- β -CD/SIM greatly induced the chondrogenic differentiation of rADSCs. Conclusively, HA- β -CD/SIM nanocomplexes will be a promising therapeutic material to alleviate inflammation as well as promote chondrogenesis.

Keywords Hyaluronic acid- β -cyclodextrin · Simvastatin · Chondrogenesis · Adipose-derived stem cells

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1 Introduction

Inflammation is closely associated with the symptoms and signs as well as progressive cartilage loss in osteoarthritis (OA). During the progression of OA, the secretion of various cytokines can be different depending on the period and severity of the disease [1]. Pro-inflammatory cytokines including IL families (i.e., IL-1 β , IL-6, and IL-17) and TNF- α are the most crucial group of molecules performing in the incidence of OA. IL-1 β has been shown to be associated with cartilage degradation and stimulation of nociceptive pathways via intracellular kinase activation [2]. Previous studies reported that IL-1 suppressed the expression of vital extracellular matrix (ECM) components including aggrecan and collagen type II in chondrocytes and stimulated the expression of proteolytic enzymes such as matrix metalloproteinases (MMPs) (i.e., MMP-1, MMP-3, and MMP-13) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (i.e., ADAMTS-4 and ADAMTS-5) from cultivated both chondrocytes and synovial fibroblasts [3, 4]. In patients with OA, the quantity of IL-1 has also been elevated in the synovial fluid, synovial membrane, subchondral bone, and cartilage [5, 6].

Statins, known as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in cholesterol biosynthesis, are the most efficient agents for cholesterol-lowering therapies such as cholestyramine and ileal bypass surgery [7, 8]. Statins have been also reported to have their cholesterol-independent pleiotropic effects such as anti-inflammatory, anti-oxidant, and immune-modulatory effects [9]. They are able to attenuate inflammation by decreasing leukocyte migration and adhesion as well as by diminishing IL-6 and IL-8 expression by vascular smooth muscle cells [10–12]. Moreover, Bellosa et al. reported that statins inhibited MMP-9 expression by activated macrophages via accumulating in atherosclerotic plaques [13]. Furthermore, the controlling effect of statins on MMPs has also been showed in a number of different cell types including smooth muscle cells, osteoblastic cells, and HT1080 fibrosarcoma cells [14, 15]. The mechanism of reduction action of statins on MMPs is reliant on the inhibition of mevalonate synthesis, resulting in production of mevalonate isoprenoid derivatives, farnesyl pyrophosphate and geranylgeranyl pyrophosphate [14, 15]. In addition to anti-inflammation effects, it was reported that simvastatin (SIM) as a mevalonate pathway inhibitor promoted chondrogenesis of intervertebral disc cells [16]. More recent study has suggested that SIM induces type II collagen expression and sulfated-proteoglycan synthesis in a dose- and time-dependent manner [17].

Based on these evidences, the potential capacity of SIM may suppress inflammatory response and induce chondrogenic differentiation. In the present study, we prepared the drug carrier for SIM long-term delivery. The drug delivery carrier was synthesized by chemical reaction of aminated hyaluronic acid (HA) with carboxylated β -cyclodextrin (β -CD) to yield HA- β -CD nanocomplexes. SIM was incorporated via inclusion into β -CD moieties in HA- β -CD. Furthermore, we evaluated whether HA- β -CD/SIM nanogels can effectively suppress inflammatory response in LPS-treated synoviocytes as well as induce chondrogenic differentiation of rat adipose-derived stem cells (rADSCs).

2 Materials and methods

2.1 Materials

Hyaluronic acid (HA, Mw = 20–100 kDa) was purchased from AllPRO Co., Ltd. (Shandong, China). Simvastatin (SIM, Mw = 418.566) was supplied from Kukjeon Pharm Co., Ltd (Anyang City, Gyeonggi-do). Ethylenediamine (EA), β -cyclodextrin (β -CD), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride (DMT-MM), succinic anhydride, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and lipopolysaccharide (LPS, from *E. coli*) were purchased from Sigma-Aldrich (MO, USA). Cellulose-ester dialysis membrane (MWCO; 12–14 kDa) was obtained from Spectrum Laboratories (CA, USA). Dubecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin–streptomycin were obtained from Gibco BRL (MD, USA).

2.2 Synthesis of HA- β -CD

For the long-term delivery of SIM, HA- β -CD was synthesized via three steps such as the synthesis of aminated HA, carboxylated β -CD, and chemical reaction between aminated-HA and carboxylated β -CD.

The introduction of amine groups into HA was performed via the chemical reaction of HA with EA in the presence of DMT-MM. HA (306 mg) and DMT-MM (141 mg) were dissolved in 100 mL of deionized water (DW) and reacted for 24 h to activate carboxyl groups of HA. Then, EA (5.46 g) was also added to the HA solution and further reacted for 2 days. Aminated HA was dialyzed using dialysis membrane (MWCO: 12–14 kDa) for 2 days and freeze-dried.

The carboxylated β -CD was synthesized as follows: β -CD (10 g) and succinic anhydride (882 mg) were dissolved in anhydrous DMF, and allowed to react under nitrogen for 2 days. After the reaction, the crude product was added

drop by drop into acetone solution at 4 °C. Then, the precipitates were collected and dried in oven at 40 °C for 2 days.

Next, HA- β -CD was synthesized by chemical reaction between aminated-HA and carboxylated β -CD. Carboxylated β -CD (200 mg) was dissolved in 20 mL of DW, and then, DMT-MM (147.2 mg) was added to activate the carboxyl group of carboxylated β -CD. After 1 h reaction, aminated-HA (100 mg) was successively added to the carboxylated β -CD solution and the mixtures were allowed to react for 2 days. Then, the resulting mixtures were dialyzed against DW for 2 days and lyophilized.

2.3 Preparation of SIM-loaded HA- β -CD (HA- β -CD/SIM) nanogels

HA- β -CD (10 mg) was dissolved in DW. SIM (1 or 10 mg) was dissolved in 1 mL of ethanol. SIM solution was carefully added dropwise at a rate of 1 mL/min in HA- β -CD solution and stirred for 1 day. The resulting solution was dialyzed against DW, and lyophilized.

2.4 Characterization of HA- β -CD and SIM-loaded HA- β -CD nanogels

The synthesized HA- β -CD was characterized with ^1H -NMR spectroscopy (500 MHz, Varian NMR spectrometer, Varian Inc., USA). The morphological shapes of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg) nanogels were determined using a transmission electron microscope (TEM, LEO912AB, Carl Zeiss, Germany) at the Korea Basic Science Institute Chuncheon Center. The particle size distributions of HA- β -CD, HA- β -CD/SIM (1 mg) and HA- β -CD/SIM (10 mg) nanogels in aqueous solution were measured using a Zetasizer 3000 instrument (Malvern Instruments, USA) that was equipped with a He-Ne laser beam at a wavelength of 633 nm, and a fixed scattering angle of 90°. To analyze the loading amount of SIM within HA- β -CD/SIM nanogels, 1 mg of HA- β -CD/SIM nanogels was clearly dissolved in DMSO and absorbance was measured at 250 nm using UV/Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

2.5 *In vitro* release of SIM

HA- β -CD/SIM nanogels (1 mg) were dispersed in 1 mL of PBS (pH 7.4) by vortexing. The dispersed nanogels were then placed in a dialysis membrane (MWCO 12–14 kDa) which was immersed in 10 mL of PBS (pH 7.4) followed by gentle shaking in a water bath (37 °C) oscillating 100 times/min. The medium was replaced with fresh medium at predetermined times. The amount of SIM released from HA- β -CD/SIM nanogels was determined at 250 nm using

UV/Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

2.6 Isolation and cultivation of rat synoviocytes and rADSCs

Synoviocytes and rADSCs were isolated from rat synovial membranes and adipose tissues, respectively (KUIACUC-2014-268 and KUIACUC-2014-5). Briefly, rat synovial tissues were isolated and rinsed with PBS. The tissues were minced with scissors and a scalpel into pieces < 3 mm and suspended in an equal volume of 0.06% (wt/v) collagenase type I (Sigma, St. Louis, MO, USA) overnight at 37 °C with gentle shaking. The tissues were mixed with an equal volume of DMEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin), followed by centrifugation at 700 rpm for 3 min at room temperature. The cells were seeded in 100-mm tissue culture plate and allowed to grow in a humidified incubator with 5% CO₂ at 37 °C.

The rADSCs were isolated to evaluate chondrogenic differentiation of HA- β -CD/SIM nanogels because ADSCs have a potential of differentiation into chondrocytes. To isolate rADSCs from rats, subcutaneous adipose tissues were removed and minced with scissors and 0.06% (wt/v) collagenase type I was added overnight at 37 °C with gentle shaking. Tissues were mixed with an equal volume of DMEM supplemented with 10% FBS and 1% antibiotics and centrifuged at 700 rpm for 3 min at room temperature. Cell pellets were filtered through a 45- μm cell strainer. The rADSCs were seeded in 100-mm tissue culture dishes and grown in a humidified incubator with 5% CO₂ at 37 °C.

2.7 Cytotoxicity test and *in vitro* cellular uptake

The cytotoxicities of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg) nanogels were evaluated against synoviocytes. Cells (5×10^4 cells/well) were seeded into 96-well culture plates and allowed to adhere for 1 day. Then, each sample (100 $\mu\text{g/mL}$) was treated to the cells. On the first and second day of incubation, the cells were washed with PBS and CCK-8 kit reagents (Dojindo, Japan) were added and incubated for 1 h. The optical density was measured at 450 nm, using a Flash Multimode Reader (VarioskanTM, Thermo, USA).

To investigate internalization of HA- β -CD nanogels into synoviocytes, FITC-NH₂ as a model compound was loaded into HA- β -CD. HA- β -CD (30 mg) and FITC-NH₂ (6 mg) were added to water (100 mL) and stirred at 300 rpm for 1 day. Then, the solution was dialyzed and lyophilized. For *in vitro* cellular uptake of HA- β -CD/FITC, synoviocytes (1×10^4 cells/well) were seeded into a Lab-Tek II

chamber slide (Nalgen Nunc International, IL, USA) and kept in DMEM including HA- β -CD/FITC (100 μ g). After 6 h incubation, the cells were rinsed with PBS and fixed in 3.7% formaldehyde for 30 min. Then, the cells were immersed in a cytoskeleton buffer (5 mM NaCl, 150 mM MgCl₂, 0.5 mM Tris-base, and 0.5% triton X-100) for 5 min at 4 °C, followed by blocking buffer (5% FBS, 0.1% Tween-20 and 0.02% sodium azide in PBS) for 30 min at 37 °C. Rhodamine-phalloidin (1:200) were added to cells for actin staining. The cells were observed by confocal laser scanning microscope (CLSM, LSM700, Zeiss, Germany).

2.8 Anti-inflammatory effects *in vitro*

The anti-inflammatory effects of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg) nanogels were investigated by determining the expressions of mRNA levels of pro-inflammatory cytokines (MMP-3, MMP-13, COX-2, ADAMTS-5, IL-6, and TNF- α) in LPS-treated synoviocytes using real-time PCR. In brief, synoviocytes (1×10^5 cells/mL) were seeded into 24-well culture plates and maintained with DMEM medium including 100 μ g/mL of each sample. After 24 h incubation, LPS (1 μ g/mL) was added to each well of culture plates and incubated for additional 3 days. At predesignated time intervals of 1 and 3 days, cells were harvested for total RNA isolation. Isolated total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's protocol. To reverse the total RNA (1 μ g) transcribed into cDNA, AccuPower RT PreMix (Bioneer, Daejeon, Korea) was used. All PCR amplifications were performed using AccuPower PCR PreMix (Bioneer, Daejeon, Korea). The primers were used as follows: MMP-3; (F) 5'-ACC TGT CCC TCC AGA ACC TG-3', (R) 5'-AAC TTC ATA TGC GGC ATC CA-3', MMP-13; (F) 5'-AAG GAG CAT GGC GAC TTC TA-3', (R) 5'-GGT CCT TGG AGT GGT CAA GA-3', COX-2; (F) 5'-CAG CCA TAC TAT GCC TCG GA-3', (R) 5'-GGA TGT CTT GCT CGT CGT TC-3', ADAMTS-5; (F) 5'-CTT CAC TGT GGC TCA CGA AA-3', (R) 5'-CAT TTG GAC CAG GGC TTA GA-3', IL-6; (F) 5'-CCG TTT CTA CCT GGA GTT TG-3', (R) 5'-GTT TGC CGA GTA GAC CTC AT-3', and TNF- α ; (F) 5'-CTC CCA GAA AAG CAA GCA AC-3', (R) 5'-CGA GCA GGA ATG AGA AGA GG-3'. PCR amplification and detection were performed on an ABI7300 Real-Time Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The relative mRNA levels of inflammatory markers were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.9 *In vitro* evaluation of chondrogenesis

2.9.1 Induction of chondrogenic differentiation

To evaluate *in vitro* chondrogenic differentiation, rADSCs were transformed to spheroid cell-type in 24-well culture plates pre-coated with agarose [18]. In brief, 50 mg of agarose was put in 50 mL bottle including DW and was melt using microwave. Dissolved agarose was coated on the surface of the inner wells of 24-well culture plates. For solidification, agarose pre-coated 24-well culture plates were carefully left for 30 min. In order to evaluate the induction of chondrogenic differentiation, the cells (1×10^5 cells/well) seeded into agarose pre-coated 24-well plates were cultivated in DMEM medium including HA- β -CD with or without SIM to form spheroid-rADSCs. After 21 days, spheroid rADSCs were harvested for further analysis.

2.9.2 Glycosaminoglycan (GAG) contents analysis

To demonstrate the chondrogenic differentiation effects of HA- β -CD/SIM on rADSCs, we evaluated GAG contents. Genomic DNA from each sample was prepared from a G-DEX™ IIc Genomic DNA Extraction Kit (iNtRon Co., Seoul, Korea) according to the manufacturer's manual. DNA contents were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA). GAG content from each sample was quantified using the dimethylmethylene blue assay. Each sample was digested in a 0.01% papain/buffer solution (100 μ g/mL in 0.1 M sodium formate, 5 mM Na₂-EDTA, and 5 mM cystein-HCl, pH 3.0) in water bath for 16 h at 65 °C. A 50 μ L aliquot of the papain digest was assayed for total GAG content by adding 250 μ L of DMB dye solution. GAG levels were determined using a Flash Multimode Reader (Varioskan™, Thermo, USA) at 595 nm.

2.9.3 Gene expression associated with chondrogenic differentiation

To investigate chondrogenic differentiation, the mRNA expression levels of the chondrogenic differentiation markers including aggrecan, collagen type II (COL2A1), and collagen type X (COL10A1) were evaluated by using a real-time PCR on day 21. Total RNA (1 μ g) isolated from each spheroid cell cluster was used for cDNA synthesis with AccuPower RT PreMix (Bioneer, Daejeon, Korea). The primer sequences for the target genes were used as follows: aggrecan; (F) 5'-TCG AGG ACA GCG AGG CC-3', (R) 5'-TCG AGG GTG TAG CGT GTA GAG A-3', COL2A1; (F) 5'-AAC CAG ATT GAG AGC ATC CG-3', (R) 5'-ACC TTC ATG GCG TCC AAG-3', and COL10A1,

(F) 5'-CAG TCA TGC CTG AGG GTT TT-3', (R) 5'-GGG TCA TAA TGC TGT TGC CT-3'. PCR amplification and detection were conducted on an ABI7300 Real-Time Thermal Cycler (Applied Biosystems, Foster, CA, USA) using a DyNamo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland). The mRNA levels of aggrecan, COL2A1, and COL10A1 were normalized to those of GAPDH and expressed as relative values.

2.9.4 Safranin-O and collagen type II staining

To further confirm the chondrogenic differentiation effects of HA- β -CD/SIM on rADSCs, the cells (1×10^5 cells/well) were seeded in a Lab-Tek II chamber slide (Nalgen Nunc International, IL, USA) and kept in DMEM containing 100 μ g of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg), respectively. At 21 days of incubation, the fixed cells with 0.1% glutaraldehyde in PBS for 20 min were washed three times with PBS and subsequently rinsed with 1% acetic acid for 10 s. Next, the cells were stained with 0.1% Safranin-O solution for 5 min, washed with PBS, and observed by a light microscope. For collagen type II staining, the cells were cultured in DMEM containing 100 μ g of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg), respectively. After 21 days, the washed cells with PBS were treated with anti-collagen type II antibody (1:500) at 4 °C under mild shaking. After overnight, goat anti-rabbit IgG (1:200) was treated to the cells for 2 h under gently shaking. Then, the cells were washed with PBS and observed with a CLSM (LSM700, Zeiss, Germany).

2.10 Statistical analysis

The data represented mean $\pm$ standard deviation, and comparisons were carried out using one-way ANOVA (Systat Software, Inc., Chicago, IL, USA). Differences were considered statistically significant at * $p < 0.05$ and ** $p < 0.01$.

3 Results

3.1 Characterization of HA- β -CD and HA- β -CD/SIM nanogels

HA- β -CD was synthesized by chemically conjugating HA with β -CD, and conjugation of aminated HA and carboxylated β -CD was confirmed by ^1H NMR spectroscopy (Fig. 1). In ^1H NMR spectrum of HA- β -CD, proton peak at 4.91 ppm was assigned to CH of β -CD. Methylene proton peaks from aminated HA and carboxylated β -CD were observed at 2.83 and 2.94 ppm, respectively. Multi peaks ranged from 3.18 to 4.2 ppm were corresponded to protons from HA or β -CD.

Figure 2A showed that HA- β -CD and HA- β -CD/SIM nanogels were amorphous powder forms and well suspended in water. Morphological shape and size distribution of HA- β -CD and HA- β -CD/SIM nanogels were characterized by TEM and Zetasizer, respectively. As shown in Fig. 2B, the morphological shapes of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg) nanogels were spherical. The determined particle sizes of HA- β -CD, HA- β -CD/SIM (1 mg), and HA- β -CD/SIM (10 mg) were

Fig. 1 A Chemical structure of HA- β -CD. B ^1H -NMR spectrum of HA- β -CD measured in D_2O

Fig. 2 **A** Photographs of HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) before and after suspension in deionized water. **B** TEM images of HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg). Scale bar: 500 nm

274.4 ± 39.8 , 350.6 ± 43.5 , and 296.9 ± 43.4 nm, respectively. The determined contents of SIM per 10 mg of HA-β-CD/SIM (1 mg) and HA-β-CD/SIM (10 mg) were 878.27 ± 39.12 and 3716.90 ± 68.18 μg, respectively.

3.2 *In vitro* cumulative release of SIM from HA-β-CD/SIM nanogels

In vitro cumulative release behavior of SIM from HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) was evaluated up to 63 days (Fig. 3). At day 1, SIM cumulatively released from HA-β-CD/SIM (1 mg) and HA-β-CD/SIM (10 mg) were 371.13 ± 0.54 μg ($42.26 \pm 0.06\%$) and 875.30 ± 3.41 μg ($23.55 \pm 0.09\%$), respectively. At day 28, SIM cumulatively released from HA-β-CD/SIM (1 mg) and HA-β-CD/SIM (10 mg) were 731.27 ± 3.42 μg ($83.26 \pm 0.39\%$) and 1757.30 ± 4.86 μg ($47.28 \pm 0.13\%$), respectively. Over the 63-day period, 826.27 ± 7.47 μg ($94.08 \pm 0.85\%$) and 2200.23 ± 9.04 μg ($59.20 \pm 0.24\%$) of SIM were cumulatively released from HA-β-CD/SIM (1 mg) and HA-β-CD/SIM (10 mg), respectively.

3.3 *In vitro* cytotoxicity study and cellular internalization

The cytotoxicity study of HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) was performed against synoviocytes at 1 and 2 days. Figure 4A showed that most

Fig. 3 *In vitro* release profiles of SIM from HA-β-CD/SIM (1 mg) and HA-β-CD/SIM (10 mg) as a function of incubation time. The SIM release was tested in PBS solutions

of the cells treated with HA-β-CD, HA-β-CD/SIM (1 mg), or HA-β-CD/SIM (10 mg) are viable. Cell viabilities in all groups were maintained over 95% at 1 and 2 days compared to that of control group, implying their low cytotoxicities to synoviocytes.

In order to evaluate cellular internalization of the nanogels, the cellular uptake of HA-β-CD/FITC was observed with CLSM. After 6 h incubation, FITC signal was observed in the cytoplasm or around the nucleus of the cells (Fig. 4B), indicating that HA-β-CD/FITC was

Fig. 4 **A** *In vitro* cytotoxicity of HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) against synoviocytes at 1 and 2 days. The data represent means $\pm$ SD ($n = 5$). **B** *In vitro* cellular internalization of HA-β-CD/FITC. Scale bar = 100 μ m

internalized into synoviocytes and internalized HA-β-CD/FITC released FITC molecules within the cells.

3.4 *In vitro* anti-inflammatory effects of HA-β-CD/SIM on LPS-treated synoviocytes

The anti-inflammatory effects of HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) were investigated by the mRNA expression levels of MMP-3, MMP-13, COX-2, ADAMTS-5, IL-6, and TNF- α in synoviocytes treated with each sample and LPS (Fig. 5). The mRNA levels of MMP-3, MMP-13, COX-2, ADAMTS-5, IL-6, and TNF- α in cells treated with HA-β-CD/SIM nanogels were decreased in a dose-dependent manner compared to those of HA-β-CD group at 1 and 3 days. Comparing with HA-β-CD/SIM (1 mg), HA-β-CD/SIM (10 mg) significantly suppressed the mRNA levels of MMP-3, MMP-13, COX-2, ADAMTS-5, IL-6, and TNF- α (** $p < 0.01$).

3.5 GAG content analysis

GAG contents from rADSC cells treated with HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) were assessed at day 21 of incubation (Fig. 6). Compared to control and HA-β-CD, GAG contents of the cells treated with HA-β-CD/SIM nanogels were significantly increased in a dose-dependent manner (** $p < 0.01$). Moreover, HA-β-CD/SIM (10 mg) significantly increased GAG content from the cells compared to HA-β-CD/SIM (1 mg) (** $p < 0.01$).

3.6 *In vitro* chondrogenic differentiation effects of HA-β-CD/SIM on rADSCs

The mRNA expression levels for aggrecan, COL2A1, and COL10A1 in rADSC cells treated on HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) were analyzed by real-time PCR after 21 days of incubation. Figure 7 showed that aggrecan, COL2A1, and COL10A1 mRNA expression in cells treated on HA-β-CD/SIM (1 mg) and HA-β-CD/SIM (10 mg) were significantly higher than those in cells treated on control and HA-β-CD (** $p < 0.01$). Aggrecan, COL2A1, and COL10A1 expression levels in cells treated with HA-β-CD/SIM (10 mg) were much higher than those in cells with HA-β-CD/SIM (1 mg) (** $p < 0.01$).

We further confirmed the chondrogenic differentiation of rADSCs treated with HA-β-CD/SIM nanogels by Safranin-O and collagen type II immunostaining. As shown in Fig. 8, in control and HA-β-CD groups, orange staining (representing proteoglycans) and red staining (representing collagen type II) were not observed. In contrast, in HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) groups, orange staining and red staining were observed in a dose-dependent manner, indicating that HA-β-CD/SIM nanogels had the potential to induce chondrogenic differentiation of rADSCs.

4 Discussion

OA is a chronic joint condition that causes pain symptoms, and it is common in the middle-aged and elderly population. There are currently no clinical interventions to cure

Fig. 5 Gene expression levels of pro-inflammatory cytokines. The gene levels of **A** MMP-3, **B** MMP-13, **C** COX-2, **D** ADAMTS-5, **E** IL-6, and **F** TNF-α in LPS-stimulated synoviocytes treated with

HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) at 1 and 3 days. The data represent mean ± SD (n = 5). * $p < 0.05$ and ** $p < 0.01$

OA. In order to alleviate the early-stage pain caused by OA, patients can only use the palliative measures such as non-steroidal anti-inflammatory drugs (NSAIDs), steroid injections, and administration of drugs or hyaluronic acid directly to the joint [19]. These methods are chronic treatments that require continued visits to the doctor, which can reduce patient compliance. In advanced stages, besides severe pain, professional and leisure activities are very limited for the patients. Patients will eventually have to undergo total joint replacement, which is major surgery and

significantly affects quality of life [20]. In order to control pain as well as prevent OA disease progression in advance, novel therapies based on drug delivery system (DDS) via intra-articular injection should be developed. In the present study, we prepared HA-β-CD/SIM nanogels and further evaluated their anti-inflammation effects on LPS-treated synoviocytes as well as chondrogenic differentiation effects on rADSCs *in vitro*.

As drug delivery carriers, HA-β-CD nanogel was fabricated via chemical conjugation of aminated HA with

Fig. 6 Glycosaminoglycan (GAG) content of rADSCs treated with HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-CD/SIM (10 mg) at 21 days. The data represent mean $\pm$ SD ($n = 5$). * $p < 0.05$ and ** $p < 0.01$

carboxylated β -CD and characterized with ^1H NMR. β -CD provides an excellent platform for ferrying many kinds of drugs because it has a unique hydrophobic interior cavity capable of incorporating hydrophobic drug molecules [21]. Due to the presence of β -CD moieties in HA- β -CD nanogel, SIM can be easily included into HA- β -CD nanogel to form inclusion nanocomplexes (HA- β -CD/SIM) whose particle sizes were ranged from approximately 300–350 nm. HA- β -CD/SIM nanogels were more effective to achieve the sustained and long-term delivery of SIM from the nanogels. This may be due to the strong interaction between hydrophobic interior cavity of β -CD and hydrophobic SIM molecules.

To evaluate *in vitro* anti-inflammatory effects of HA- β -CD/SIM nanogels, synoviocytes made inflammatory cells by LPS treatment [22]. It is well-known that LPS as lipoglycans and endotoxins is a major component of the outer membrane of Gram-negative bacteria. Our previous study showed that LPS-treated synoviocytes increased the mRNA expression levels of inflammatory cytokines such

Fig. 7 Gene expression levels of chondrogenic differentiation markers. The gene levels of **A** aggrecan, **B** COL2A1, and **C** COL10A1 in rADSCs treated with HA-β-CD, HA-β-CD/SIM (1 mg), and HA-β-

CD/SIM (10 mg) at 21 days. The data represent mean $\pm$ SD ($n = 5$). * $p < 0.05$ and ** $p < 0.01$

Fig. 8 Chondrogenic differentiation effects of HA- β -CD/SIM nanogels on rADSCs. **A** Safranin-O staining. **B** Collagen type II immunostaining. Scale bar = 100 μ m

as MMP-3, MMP-13, COX-2, ADAMTS-5, IL-6, and TNF- α [22]. The cells cultured with HA- β -CD showed the enhanced expression of these inflammatory markers at 1 and 3 days after LPS treatment, indicating that HA- β -CD had no anti-inflammatory effects. However, the cells cultured with HA- β -CD/SIM nanogels showed markedly decreased the expression of these inflammatory cytokines at the same time points. As shown in drug release study and cellular internalization of the nanogels, anti-inflammatory effects of HA- β -CD/SIM nanogels on LPS-treated cells might be due to the action of the released SIM molecules from the nanogels following the cellular internalization of HA- β -CD/SIM nanogels, and thus, HA- β -CD/SIM nanogels effectively reduced inflammation in LPS-treated synoviocytes. Previous study showed that SIM is effective to reduce the expression levels of IL-6, TNF- α , and MMP-9 in human fetal membranes [23]. McFarland et al. reported that statins such as lovastatin, simvastatin, pravastatin, atorvastatin, pitavastatin, and fluvastatin could significantly reduce IL-1 β , TNF- α , and prostaglandin E2 (PGE2) in LPS-stimulated microglial-like cells [24]. In another study, SIM dose-dependently inhibited IL-6, IL-8, and nitric oxide (NO) production of isolated chondrocytes and cartilage which were stimulated with a mixture of IL-1 β and TNF- α [25]. Coincidental with previous results, we also exhibited that HA- β -CD/SIM nanogels effectively reduced the expressions of diverse pro-inflammatory cytokines in LPS-induced synoviocytes, indicating that HA- β -CD/SIM nanogels are effective in preventing inflammation reactions.

Chondrogenic differentiation of rADSCs treated with HA- β -CD/SIM nanogels was evaluated in the 3D chondrogenic culture systems by measuring GAG content which is a main matrix element of the cartilage and a marker for

chondrogenic differentiation [26]. The GAG content of rADSCs treated with HA- β -CD showed similar levels compared to that of rADSCs alone, indicating that HA- β -CD nanogels without SIM have not induced chondrogenic differentiation. It has been reported that HA scaffold enhances cell to cell contact, and interactions between HA scaffold and various cell-surface receptors including CD44 play an important role in maintaining differentiated characteristics of chondrocytes [27]. In the present study, HA- β -CD nanogels have no chondrogenic effect on rADSCs because the nanogel systems may not enhance cell to cell contact when compared to HA scaffold system. Previous studies reported that statins could block cartilage collagen breakdown, induced by pro-inflammatory cytokines, via the downregulation of cartilage-degrading enzymes [28, 29]. Consistent with the previous studies, this study also showed that HA- β -CD/SIM nanogels significantly and dose-dependently decreased pro-inflammatory cytokines and cartilage-degrading enzymes. Due these features, HA- β -CD/SIM nanogels increased the GAG content and induced chondrogenic differentiation of rADSCs.

To further evaluate the chondrogenic differentiation effects of rADSCs treated with HA- β -CD/SIM nanogels, we analyzed the mRNA levels of aggrecan, collagen type II (COL2A1), and collagen type X (COL10A1). Aggrecan and type II collagen are the predominant proteoglycan and collagen fiber of the cartilage, respectively. Thus, their mRNA expressions have been generally considered as the primary marker for chondrogenic differentiation [30, 31]. COL2A1 is a cartilage-specific gene that encodes the type II collagen α -1 chain and a cartilage-specific marker [32]. Moreover, the increase in the mRNA expression level of type X collagen is observed uniquely in the differentiation of mature chondrocytes into hypertrophic chondrocytes

[33]. When stimulated by chondrogenic differentiation of mesenchymal stem cells (MSCs), the expression of aggrecan, COL2A1, and COL10A1 is well known to be upregulated [34, 35]. The mRNA expression levels of aggrecan, COL2A1, and COL10A1 in rADSCs treated with HA- β -CD/SIM nanogels were markedly higher than those in rADSCs alone or in rADSCs treated with HA- β -CD. Recently, Han et al. has suggested that SIM induces type II collagen expression and sulfated-proteoglycan synthesis in a dose- and time-dependent manner [17]. Also, they showed that SIM induces differentiation of rabbit articular chondrocytes via ERK-1/2 and p38 kinase pathways. According to our results, the increase in the mRNA expression levels of aggrecan, COL2A1, and COL10A1 indicated that rADSCs have been differentiated to chondrocytes. Moreover, Safranin-O and collagen type II staining data supported that HA- β -CD/SIM nanogels had the potential to induce chondrogenic differentiation of rADSCs in a dose-dependent manner.

Consistent with the previous studies, our results corroborated that the delivery of SIM using HA- β -CD nanogels suppresses inflammatory response in LPS-stimulated synoviocytes as well as enhances chondrogenic differentiation of rADSCs. In further study, we will focus on *in vivo* study to demonstrate that HA- β -CD/SIM nanogels may be a potential therapeutic drug for OA therapy.

In conclusion, HA- β -CD/SIM nanogels were prepared by chemical reaction of aminated HA with carboxylated CD and inclusion of SIM into hydrophobic interior cavity of β -CD moieties in HA- β -CD. HA- β -CD/SIM formed inclusion nanocomplexes with 300–350 nm diameter. Due to strong interaction between interior cavity of β -CD moieties and SIM, HA- β -CD/SIM nanogels showed sustained and long-term release behavior of SIM from nanogels. *In vitro* studies demonstrated that HA- β -CD/SIM nanogels significantly suppressed the mRNA expression levels of MMP-3, MMP-13, COX-2, ADAMTS-5, IL-6 and TNF- α in LPS-stimulated synoviocytes. Moreover, HA- β -CD/SIM nanogels greatly induced chondrogenic differentiation of rADSCs by elevating the chondrogenic markers such as GAG content, aggrecan, COL2A1, and COL10A1. This study suggests that HA- β -CD/SIM nanogels will be a promising therapeutic material capable of reducing inflammation and inducing chondrogenesis for OA treatment.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical statement Animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the Korea University Medical Center (KUIACUC-2014-268 and KUIACUC-2014-5).

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