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Effects of sesamin on the biosynthesis of chondroitin sulfate proteoglycans in human articular chondrocytes in primary culture

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Abstract Osteoarthritis (OA) is a degenerative joint disease that progressively causes a loss of joint functions and the impaired quality of life. The most significant event in OA is a high degree of degradation of articular cartilage accompanied by the loss of chondroitin sulfate-proteoglycans (CS-PGs). Recently, the chondroprotective effects of sesamin, the naturally occurring substance found in sesame seeds, have been proved in a rat model of papain-induced osteoarthritis. We hypothesized that sesamin may be associated with possible promotion of the biosynthesis of CS-PGs in human articular chondrocytes. The aim of the study was to investigate the effects of sesamin on the major CS-PG biosynthesis in primary human chondrocyte. The effects of sesamin on the gene expression of the PG core and the CS biosynthetic enzymes as well as on the secretion of glycosaminoglycans (GAGs) in monolayer and pellet culture systems of articular chondrocytes. Sesamin significantly increased the GAGs content both in culture medium and pellet matrix. Real-time-quantitative PCR showed that sesamin promoted the expression of the genes encoding the core protein (*ACAN*) of the major CS-PG aggrecan and the biosynthetic enzymes (*XYLT1*, *XYLT2*, *CHSY1* and *CHPF*) required for the synthesis of CS-GAG side chains. Safranin-O staining of sesamin treated chondrocyte pellet section confirmed the high degree of GAG accumulation. These results were correlated with an increased level of secreted GAGs in the media of cultured articular chondrocytes in both culture systems. Thus, sesamin would provide a potential therapeutic strategy for treating OA patients.

Keywords Sesamin · Human articular chondrocytes · Chondroitin sulfate · Proteoglycan · Glycosaminoglycan · Osteoarthritis

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Introduction

Osteoarthritis (OA) is the most important joint diseases in the field of orthopedics and occurs worldwide with the highest prevalence. Moreover, OA is associated with an extremely high economic burden, which is largely attributable to the effects of disability and the expense of the treatments as well as the impaired quality of life [1]. The disease is characterized by a progressive loss of articular cartilage that causes chronic pain and compromises joint function. These events are accompanied by changes in a complex network of biochemical factors, including proteolytic enzymes, which cause a breakdown of the cartilage macromolecules especially collagen and a major chondroitin sulfate (CS)-proteoglycan (PG), aggrecan [2, 3]. Several studies recently reported that the degradation of CS-PGs was correlated with the severity of OA [4]. The current OA treatment is primarily focused on a symptomatic

relief by the use of rapidly acting drugs, especially NSAIDs (non-steroidal anti-inflammatory drugs) [5]. NSAIDs increase the risk of upper gastrointestinal adverse effects and does not affect the underlying pathogenesis of the disease [6]. Hence, there has been a continuous search for new and better drugs for OA. There has been increase in the use of symptomatic slow acting drugs such as glucosamine, CS and diacetylrhein (diacerein [7]. These drugs could favor anabolic processes in the OA cartilage and contribute to a delayed progression of the disease *via* inhibition of the catabolic processes [8]. Currently, there are very few safe drugs or innovations that have been proved to restore cartilage or curtail the disease processes [9]. Thus, truly disease-modifying and safer agents for the management of OA are still desired. Many studies have shown that alternative herbal medicines or phytochemicals have possible chondroprotective properties and are interesting candidates for the non-pharmacological intervention. Thus, herbal medicines may be suitable candidates for OA treatment.

Sesame (*Sesamum indicum* L.) has been used, extensively, as a traditional food in eastern countries. Sesame seeds and oil are widely used in cooking [10]. Sesame seeds contain a group of compounds called lignans, which are one of the major classes of phytoestrogens, and play an important role in health-promoting effects. They have the pharmacological properties including anti-inflammatory effects [11], antioxidant activity [12], antihypertensive effects [13], apoptosis-induction in tumor cells [14], enhancing antioxidant activity of vitamin E [15], lowering cholesterol levels [16], improving fatty acid metabolism [17], and neuroprotective effects against hypoxia [18]. Recently, we reported that sesamin treatment *in vitro* reversed pathological changes in OA cartilage: reduced the disorganization of chondrocytes in cartilage, increased cartilage thickness and decreased losses of type II collagen and CS-PGs [11].

In the past decade, there has been no study related to chondroprotection that has focused on the effects on the genes of biosynthesis of CS-PGs. Therefore, here we studied using cultured HAC (human articular chondrocytes) systems, the effects of sesamin on the gene expression of the core protein of the major CS-PG, aggrecan and on the glycosyltransferases required for the attachment of CS side chains on the aggrecan core protein. Aggrecan core protein is encoded by the *ACAN* gene, which is highly expressed in cartilagenous tissues [19–22]. *XYLT1* and *XYLT2* encode xylosyltransferase I (XylT1) and xylosyltransferase II (XylT2), respectively, which catalyze the initial enzymatic reaction in the assembly of GAGs onto core proteins. This addition of a xylose to specific serine residues in the core protein is a rate-limiting step for aggrecan biosynthesis [19, 23, 24]. *CHSY1* and *CHPF* encode chondroitin synthase 1 (ChSy1) and chondroitin polymerizing factor (ChPF), respectively. Their heterodimeric complex exhibits chondroitin polymerase activity [25, 26], which extends the CS chain by adding alternate GalNAc and GlcUA sugar residues from nucleotide sugar substrates. To investigate effects of sesamin on the expression of the genes encoding aggrecan (ACAN) and CS biosynthetic enzymes including XYLT1, XYLT2, CHSY1, and CHPF as well as the content of GAGs, two different HAC culture systems, monolayer and pellet cultures, were utilized in this study. It is notable that full production of CS-GAGs is essential for healthy cartilage development as genetic defects of certain glycosyltransferases and sulfotransferases involved in the biosynthesis of CS-GAGs cause developmental delay, short stature, generalized osteopenia, chondrodysplasia, craniofacial dysmorphism, and OA [22, 27].

Material and methods

Chemicals

CS-C and the Hoechst 33258 dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). Custom primers for RT-qPCR were purchased from Bio-Rad (Hercules, CA, USA). illustra RNAspin Mini RNA Isolation Kit was purchased from GE Healthcare (Buckinghamshire, UK). iScript™ Reverse Transcription Supermix and SsoFast™ EvaGreen® Supermix were purchased from Bio-Rad (Hercules, CA, USA).

Sesamin was prepared from *S. indicum* L. by extraction with hexane and column chromatography on silica gel as described by T. Phitak *et al.* [11]. The structure of the purified material was examined and identified by nuclear magnetic resonance spectroscopy and mass spectrometry. Furthermore, co-chromatographic analysis was carried out with the authentic sesamin (Sigma-Aldrich®) for its purity. The mass spectrogram of the purified preparation and chemical structure of sesamin are shown in Fig. 1.

Cells

HAC from non-osteoarthritis joints were harvested from articular cartilage of 18–45 year-old patients, and chondrocytes were isolated according to a standard protocol. Informed consents were obtained from all the patients, and all procedures of the chondrocyte isolation were approved by the Ethical Committee of Faculty of Medicine, Chiang Mai University (approval no. 070CT111016).

Detroit 551 is a human skin fibroblast cell line purchased from ATCC® (CCL-110). Detroit 551 was used as a negative control cell line because the main CS-PG of Detroit 551 is versican but not aggrecan [28].

Cell culture and treatment

The original morphology of HAC is a spherical shape. However, after isolation, their *in vitro* expansion is intrinsically

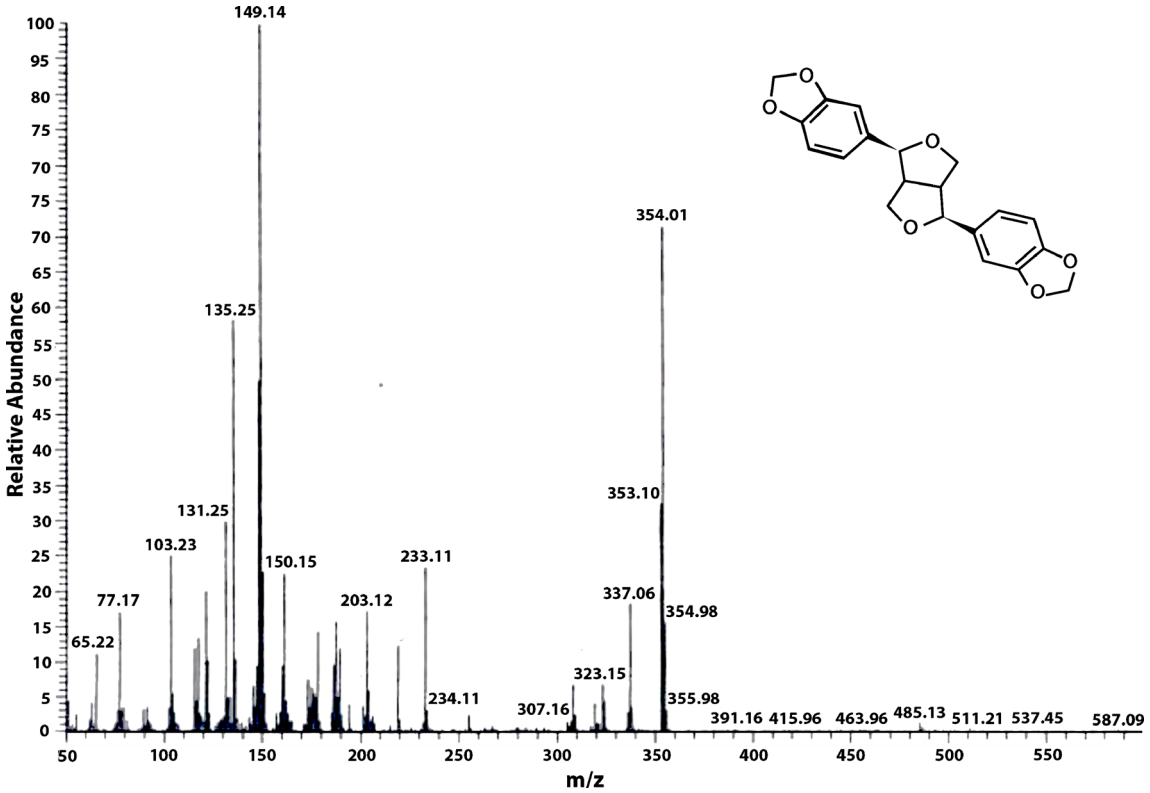

Fig. 1 The mass spectrogram and chemical structure of the sesamin preparation purified from *S. indicum* L.

associated with cellular de-differentiation and reduced ability to re-differentiate. The de-differentiation occurs when chondrocytes are cultured under conditions allowing them to attach and spread on a two-dimensional (2D) surface in a monolayer culture. In this environment, chondrocytes gradually lose their spherical shape and acquire an elongated fibroblast-like morphology. These morphologic alterations are accompanied by profound biochemical changes, as indicated by the reduction or total loss of aggrecan and the synthesis of type II collagen with the increased synthesis of versican CS-PG and type I collagen. However, de-differentiated chondrocytes have the capacity to re-differentiate when transferred into an environment supporting a spherical morphological formation (a pellet culture) [29].

For monolayer cultures, 4.5×10^4 cells/well were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal calf serum (FBS). Cells were maintained in culture in a humidified incubator with 5 % CO_2 at 37 °C. The culture medium was collected for measuring GAGs.

For pellet cultures, 5.0×10^5 cells/pellet were formed with 1 ml medium in a 15 ml conical culture tube by being spun for 5 min at 160× g, and were grown as cell pellets in the medium: 10 % FCS/DMEM, Insulin-Transferrin-Selenium (ITS 1×), 25 μg/ml ascorbic acid 2-phosphate and 10^{-7} M dexamethasone. Pellets were allowed to grow in a humidified incubator (37 °C, 5 % CO_2) for 21 days with regular medium changes (every 2–3 days). The culture medium was collected for measuring GAGs, whereas the cell pellets were digested with papain to determine the content of GAGs and DNAs. GAGs and DNAs in each culture medium and the supernatant fluid of the papain digest of the pellet were quantified using 1,9-dimethylmethylene blue (DMMB), whereas DNAs in the papain digests were determined using Hoechst 33258 as described below.

Methylthiazole tetrazolium (MTT) assay

1.0×10^4 cells were plated in triplicate in 96-well plates and incubated overnight. The cells were treated with various concentrations of sesamin (0–10 μM) for 24 h. After incubation, culture media were discarded and replaced with 100 μl 0.5 mg/ml MTT for 4 h. The MTT agent was discarded and 100 μl of dimethyl sulfoxide was added in each well to solubilize the formazane crystals. The absorbance was measured at 540 nm using a microplate reader. Percent of survived cells was calculated as follows: Percentage of survival=(OD. of sample×100)/OD. of control.

Quantification of GAGs with DMMB

GAGs were determined using DMMB with CS-C (0–60 μg/ml) as a standard. 200 μl of a DMMB solution was added to 50 μl of a sample (a conditioned medium or a supernatant fluid recovered from a papain digest of the cell pellet) and the

standard prior to reading absorbance values at 525 nm in a spectrophotometer for microplates.

Quantification of DNA using Hoechst 33258

Papain-digested individual samples (20 μl each) were added to 2 ml of a dye/buffer solution, and the fluorescence of the samples was measured using the excitation and emission wavelengths of 450 and 555 nm, respectively. A standard curve was generated using known concentrations of standard DNA (0–200 ng/ml).

Reverse transcribed quantitative–polymerase chain reaction (RT-qPCR)

RT-qPCR was used to examine the gene expression in the monolayer and pellet cultures of HAC treated with or without sesamin. Total RNA was isolated using an RNA extraction kit following the manufacturer's protocol (GE Healthcare). Total RNA (1 μg) was converted to cDNA using iScript™ Reverse Transcription Supermix. For determination of the gene expression, the reaction setup of SsoFast™ EvaGreen® Supermix and Real-Time PCR system was used. Gene expression was measured in triplicate in three separate experiments and relative gene expression was converted using the $2^{-\Delta\Delta ct}$ method [30] against the internal control *GAPDH* as a house-keeping gene. Primer sequences are shown in Table 1.

Hematoxylin-eosin (H&E) and Safranin-O stainings

The pellets obtained at day 21 were collected in phosphate-buffered saline containing 0.4 % formaldehyde at 4 °C. The pellets were processed into paraffin wax blocks and sectioned. Four-micrometer sections were stained with H&E for assessment of cell/tissue morphology and with Safranin-O for the localization of GAGs. The stained sections were mounted on glass slides and were observed at 400× magnification by light microscopy.

Table 1 Real-Time PCR primer sequences

Genes	Primer sequence (5′-3′)
ACAN	Forward: ACTTCCGCTGGTCAGATGGA
	Reverse: TCTCGTGCCAGATCATCACC
XYLT1	Forward: GTGGATCCCGTCAATGTCATC
	Reverse: GTGTGTGAATTCGGCAGTGG
XYLT2	Forward: CGAATCGCCTACATCATGCTGG
	Reverse: TAAACGGCCTTGAGGAGACG
CHSY1	Forward: CCCGCCCCAGAAGAAGTC
	Reverse: TCTCATAAACCATTCATACTTGTCCAA
CHPF	Forward: AGATCCAGGAGTTACAGTGGGAGAT
	Reverse: CCGGGCGGGATGGT
GAPDH	Forward: GAAGGTGAAGGTCGGAGTC
	Reverse: GAAGATGGTGATGGGATTTC

Statistical analysis

All values are given as mean ± standard deviation from triplicate samples of three independent experiments. The non-parametric test (Mann–Whitney *U* test) was used to compare treated and untreated control cells. Differences were considered statistically significant when $p < 0.05$ and $p < 0.01$.

Results

Investigation of cytotoxic effects of sesamin on primary HAC and Detroit 551 cells

Prior to an investigation of the effects of sesamin on the biosynthesis of CS-PGs by HAC, the cytotoxicity of sesamin was first examined. The presence of sesamin at concentrations (0.16–10 μM) did not affect cell viability (>80 %) compared with untreated control cells. Therefore, based on the methylthiazole tetrazolium (MTT) assay, sesamin at these concentrations was not toxic to either HAC or Detroit 551 cells (data not shown).

Time-course characterization of the expression of the genes encoding aggrecan (ACAN) and CS biosynthetic enzymes (*XYLT1*, *XYLT2*, *CHSY1* and *CHPF*)

Cultured HAC treated with sesamin for 24 h were collected at 12 time points and cell lysates were used for RNA extraction and gene expression analysis by RT-qPCR. At 45 min of treatment, 2 μM sesamin significantly up-regulated the expression of *ACAN*, *XYLT1*, *XYLT2*, *CHSY1* and *CHPF* with 2.56, 2.50, 3.11, 2.71 and 2.37-fold, respectively, when compared with untreated cells (data not shown). Moreover, the expression of these genes in HAC cells treated with sesamin for 5, 6 and 20 h was similarly up-regulated (data not shown). The 45-min time point was then chosen for the treatment in the further experiments because it was the earliest time point showing a significant change of the gene expression encoding ACAN and CS biosynthetic enzymes.

Investigation of the effects of sesamin on the expression of the genes involved in the synthesis of CS-PGs in HAC and Detroit 551 cells

In monolayer HAC cultures, it was found that the treatment with sesamin at a high dose (2 μM) could significantly increase the expression of *ACAN*, *XYLT1*, *XYLT2*, *CHSY1* and *CHPF* genes with 2.62, 2.36, 3.00, 2.96, and 2.41-fold, respectively, when compared with untreated cells (Fig. 2a–e). Moreover, sesamin selectively increased the expression of all

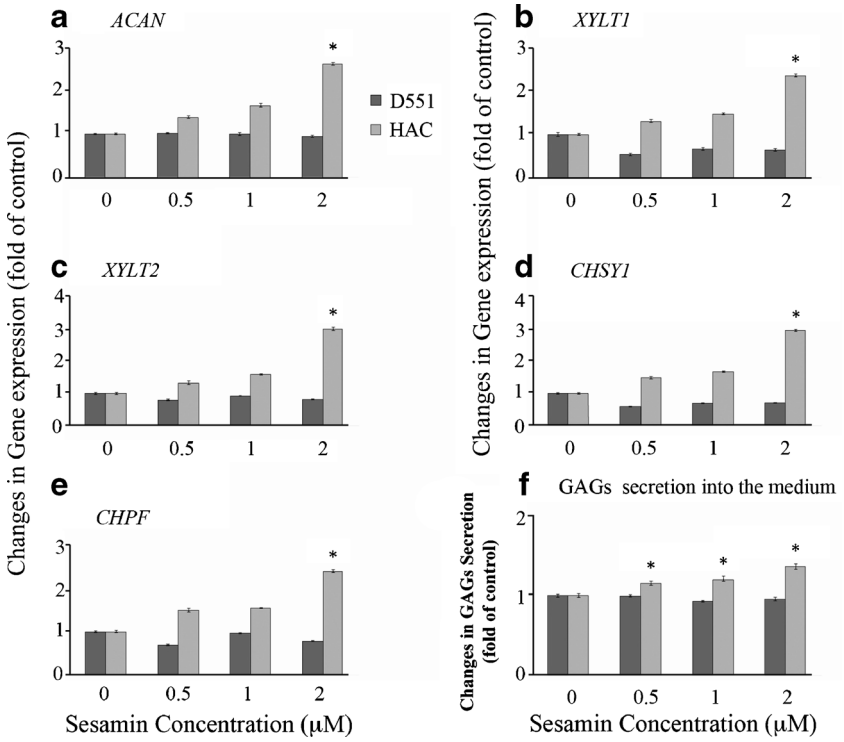

Fig. 2 The effects of sesamin on the expression of the genes involved in the synthesis of CS-PGs and the secretion of GAGs. HAC and D551 cells were cultured in a monolayer system and treated with various concentrations of sesamin (0–2 μM) for 45 min. The expression of the genes for the synthesis of CS-PGs: *ACAN* (**a**), *XYLT1* (**b**), *XYLT2* (**c**), *CHSY1* (**d**) and *CHPF* (**e**) was analyzed by RT-qPCR. GAGs released by sesamin-treated HAC and D551 cells. The cells were treated with sesamin (0.5–2 μM) for 45 min. The conditioned media were collected and analyzed for GAGs by the DMMB assay (**f**). Each value is expressed as mean ± standard deviation of 3 independent analyzes (see "Methods"), * = $p < 0.05$ in sesamin-treated vs untreated cells

tested genes involved in the biosynthesis of CS-PGs in a dose dependent manner only in HAC, but not in Detroit 551 cells. Detroit 551 cells were used as control cells because we were interested in investigating the effects of sesamin on the gene expression of aggrecan CS-PG and the CS biosynthetic enzymes, whereas the Detroit 551 cell line is derived from normal human skin and expresses versican CS-PG as the predominant PG, but not aggrecan [28, 31]. Consistent with the up-regulation of the CS synthesizing enzymes, the amount of GAGs in the conditioned media of the monolayer HAC cultures after treatment with 1 and 2 μM sesamin on day 21 was 1.3 and 1.5-fold, respectively, as compared with that of the untreated cell (Fig. 2f).

In pellet cultures, HAC and Detroit 551 cells were cultured in 3D (5×10^5 cells per pellet) in chondrogenic media and treated with 0.5, 1 and 2 μM sesamin for 21 days. Conditioned media and pellets were collected every 3 and 7 days for analyzing GAGs (on day 1, 4, 7, 10, 14, 16, 19 and 21) and the gene expression (on day 1, 7, 14 and 21), respectively.

The gene expression of proteins involved in the CS biosynthesis was determined every 7 days during the treatment of the HAC pellet culture for 21-days with sesamin. Sesamin (at 1 and 2 μM) up-regulated the expression of all tested genes involved in the CS synthesis on day 14. The expressions of *ACAN*, *XYLT1*, *XYLT2*, *CHSY1* and *CHPF* at 1 μM sesamin increased 1.89, 2.66, 2.30, 3.29, and 3.07-fold, respectively, compared with untreated cells. Upon treatment with 2 μM sesamin, the expression of these genes increased 3.00, 3.34, 3.79, 5.99, and 3.66-fold, respectively, when compared with untreated cells (Fig. 3). In addition, under some conditions of the sesamin treatment, for example on day 7, a significant increase was observed in the expression of the following genes encoding the CS biosynthetic enzymes: *XYLT2* at 2 μM sesamin (1.8-fold of untreated cells) (Fig. 3c) and *CHSY1* at 1 and 2 μM sesamin (3.5 and 6.0-fold of untreated cells, respectively) (Fig. 3d). On day 21, however, the expression of the genes encoding these enzyme proteins showed no significant difference between sesamin-treated and untreated pellet cultures (data not shown). Notably, sesamin showed the specific increase in the expression of the genes encoding the enzyme proteins for CS biosynthesis in HAC cultures, whereas it exhibited no significant effect on the pellet culture of the Detroit 551 cells (Fig. 3a–d)

Investigation of the effects of sesamin on GAGs secreted in the culture media

Since we previously showed that sesamin can maintain the thickness of OA-cartilage and give high density of Safranin-O staining in an OA-induced rat model [11], the effects of sesamin on the secretion of GAGs was investigated in the HAC culture media. In monolayer cultures, it was found that sesamin (at 0.5, 1.0 and 2.0 μM) could significantly increase the secretion of GAGs into the culture media of HAC 1.2, 1.2, and 1.4-fold, respectively, when compared with untreated cells (Fig. 2f). These results correlated well with those of the gene expression of the CS biosynthetic enzymes tested. In contrast, sesamin had no effect on the Detroit 551 cell line.

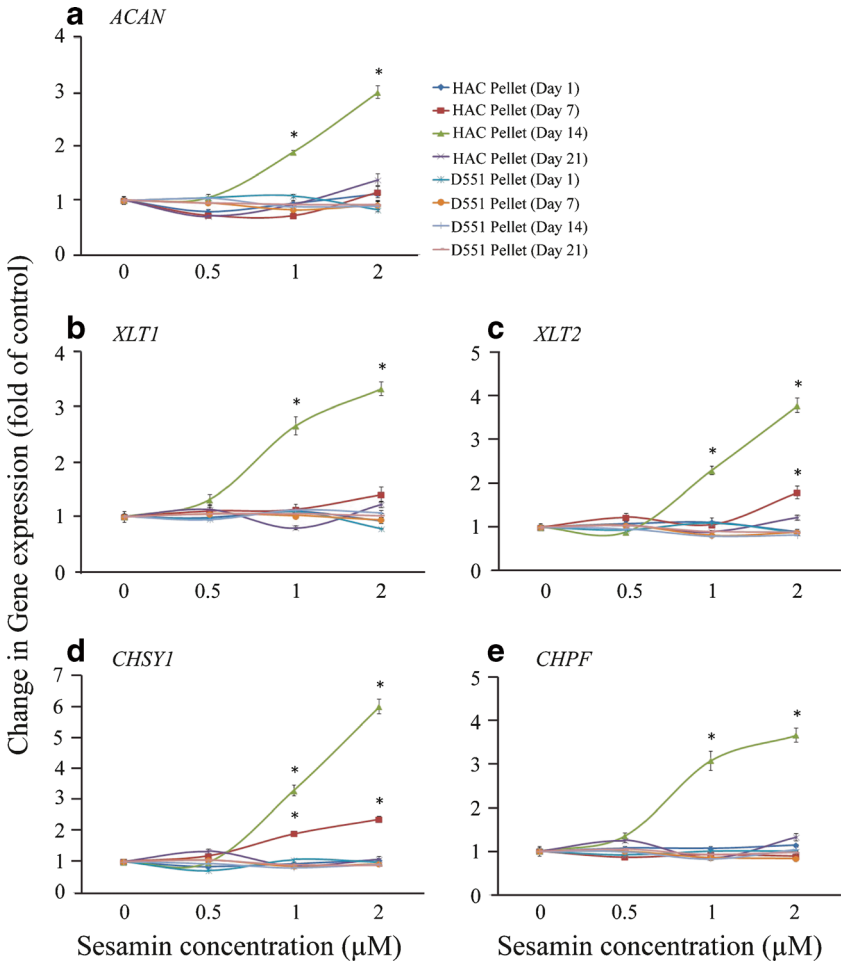

Fig. 3 The effects of sesamin on changes in the expression of CS-PGs synthesis involved genes during the treatment for 21 days. HAC and D551 pellets were treated with various concentrations of sesamin (0.5, 1 and 2 μM) for 21 days. The total RNA was extracted on days 1, 7, 14 and 21 for analyzing the gene expression. The expression of *ACAN* (**a**), *XYLT1* (**b**), *XYLT2* (**c**), *CHSY1* (**d**) and *CHPF* (**e**) was analyzed by RT- qPCR. Each value is expressed as mean ± standard deviation of 3 independent analyzes (see "Methods"), * = $p < 0.05$ in sesamin-treated vs untreated cells

Pellets of HAC and D551 cells were cultured in media for 21 days as described in "Experimental Procedures". The chondrogenic media obtained from the pellets were changed every 3 days, and collected for the measurement of the level of GAGs secreted into the media. The accumulation and variation of GAGs in both types of the cells were analyzed by the DMMB assay. The DNA content (μg/ml) was quantified using Hoechst 33258 as an index of the cell number to normalize each GAG value.

For the pellet culture of Detroit 551 cells, the sesamin treatment gave no significant effect on the accumulation of GAGs in the culture media compared to the untreated cells. In contrast, in the HAC pellets cultured for 10 days, treatments with 1 and 2 μM sesamin significantly increased the accumulation of GAGs in the media 1.3-fold as compared with the untreated cells (Fig. 4a). The secretion of GAGs into the media was also significantly raised between days 10–16 (Fig. 4c and d). Sesamin at the concentrations of 1 and 2 μM caused an increase in the GAG level in the media as follows: 1 μM sesamin on days 10, 14, and 16 increased the GAG level 1.6, 1.7 and 1.3-fold, respectively, as compared with untreated cells (Fig. 4c). Sesamin at 2 μM on days 10, 14 and 16 increased the GAG level 1.5, 1.6 and 1.3-fold, respectively, as compared with the untreated cells (Fig. 4d). In addition, the increase in GAGs at 1 and 2 μM sesamin on day 14 was correlated with the increase in the expression of the genes encoding the CS biosynthetic enzymes on day 14 (Fig. 3).

Sesamin had no significant effect on the secretion of GAGs in the conditioned media of the pellet-cultured Detroit 551 cells (data not shown). GAGs in the collected conditioned media recovered from the pellet cultures of HAC and Detroit 551 cells were determined by the DMMB assay and the DNA content (μg/ml) in each cell pellet was measured by the Hoechst assay to normalize the GAG values relative to the cell number. The results indicated that the amount of GAGs in the conditioned media of the HAC pellet after treatment with 1 and 2 μM sesamin on day 21 was 1.5 and 1.6-fold, respectively, as compared with that of the untreated cell (data not shown). Moreover, treatments with 1 and 2 μM sesamin showed an elevation of the accumulated GAGs on day 21, 1.5 and 1.7-fold, respectively (data not shown), when compared with those observed on day 1 after treatment with 1 and 2 μM sesamin.

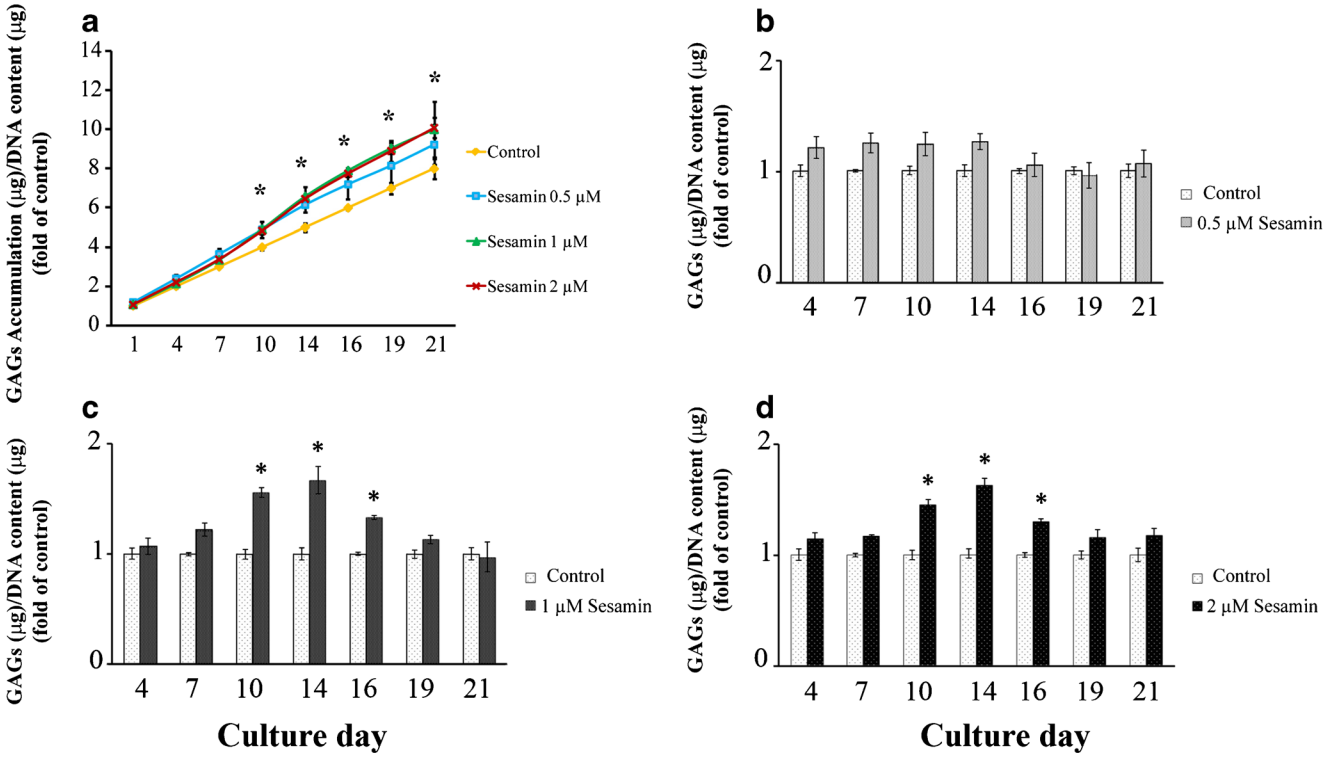

Fig. 4 The effects of sesamin on the GAGs secretion during the treatment for 21 days. HAC pellets were treated with 0.5, 1 and 2 μM sesamin for 21 days. GAGs detected by the DMMB assay after treatments with 0.5 (**b**), 1 (**c**) and 2 μM (**d**) are shown. The accumulation (**a**) and variation of GAGs in each concentration were as follows: *asterisk* in figure **a** = $p<0.05$ in 1 and 2 μM sesamin-treated vs untreated cells, respectively. * = $p<0.05$ in sesamin-treated vs untreated cells (**c**, **d**)

Investigation of the effects of sesamin on the accumulation of GAGs in the pellet matrix

GAGs are not only secreted into the culture medium but also accumulate in the pellet matrix. The GAGs in the pellet were analyzed after papain digestion at 60 °C for 18 h in order to remove proteins. On days 1 and 21, the papain digest supernatants were collected by centrifugation from HAC and Detroit 551 pellets for measurement of accumulated GAGs. Results were normalized to the cell number following assay of the DNA content (μg/ml) in each digest.

The results showed that sesamin had no significant effect on accumulation of GAGs in the matrix of the Detroit 551 cell pellet (Fig. 5a). In contrast, the GAG level in the HAC pellet treated with 1 and 2 μM sesamin increased on day 21, 1.9 and 2.2-fold, respectively (Fig. 5b), when compared with the untreated HAC pellet. Moreover, treatments with 1 and 2 μM sesamin increased the GAG level on day 21, 2.3 and 2.6-fold, respectively (Fig. 5b), when compared with that observed on day 1 after treatment with 1 and 2 μM sesamin.

Effects of sesamin on the accumulation of GAGs in the matrix

The Detroit 551 cell pellets and HAC pellets on day 21 were sectioned and stained using hematoxylin and eosin (H&E) as well as Safranin-O for examining the cell morphology and the

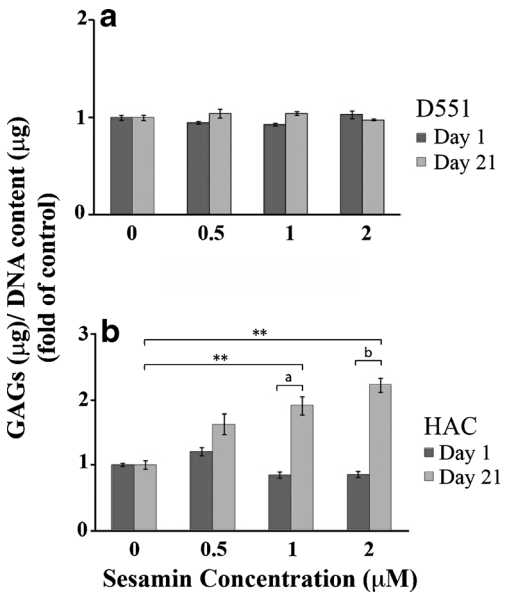

Fig. 5 The effects of sesamin on the accumulation of GAGs in the pellet matrix. Pellet cultures of D551 cells (**a**) and HAC (**b**) were treated with 0.5–2 μM of sesamin for 21 days. On days 1 and 21, the supernatant fluid of a papain digest of the cell pellets were collected to analyze GAGs accumulated in the matrix by the DMMB assay. **a** = $p<0.01$ in 1 μM sesamin treatment on day 21 *vs* 1 μM sesamin on day 1. **b** = $p<0.01$ in 2 μM sesamin treatment on day 21 vs 2 μM sesamin treatment on day 1. ** = $p<0.01$ in sesamin-treated vs untreated cell pellets

accumulation of GAGs, respectively. H&E staining showed the morphology characteristic of Detroit 551 skin fibroblasts (data not shown) and chondrocytes (Fig. 6a) in the pellet cultures. It was found that treatments with the sesamin concentrations of 0.5, 1 and 2 μM did not affect the morphology of the Detroit 551 cells when compared with the untreated controls (data not shown). Sesamin treatments at the concentrations of 0.5, 1 and 2 μM did not affect the HAC morphology when compared with the untreated controls (Fig. 6a). Thus, sesamin had no effects on the morphology of either HAC or Detroit 551 cells under the pellet culture conditions.

The GAGs of both fibroblasts and chondrocytes were found to be embedded in the extracellular matrix of the cultured pellets of the Detroit 551 cells (data not shown) and HAC (Fig. 6b). Safranin-O staining revealed that sesamin had no effects on the accumulation of GAGs in the cultured Detroit 551 cell pellet (data not shown). In strong contrast, the HAC pellet cultured at the sesamin concentrations of 1 μM and 2 μM markedly increased the intensity of red color in the matrix area of the pellet sections when compared with the untreated counterparts (Fig. 6b), confirming the accumulation of extracellular GAGs. Thus, the GAG analysis and staining showed that sesamin increased GAG production in the HAC pellet.

Discussion

Osteoarthritis (OA) is a progressive degenerative joint disease characterized by cartilage degeneration and an imbalance between the synthesis and degradation of the CS-PG aggrecan, leading to an impairment of joint functions and quality of life. The burden of the disease dramatically impacts health leading to joint replacement [1, 9]. According to the adverse effects of the rapid-action drugs for symptomatic relief, there has been an increased use of symptomatic slow acting drugs (chondroprotective drugs or connective tissue structure modifying agents) such as chemically modified tetracyclines, glucosamine (chondroitin sulfate) and diacetylrhein (diacerein) [32, 33]. However, glucosamine is a dietary supplement not a pharmaceutical drug. The most common side effect of the diacerein treatment is diarrhea [34, 35]. Thus, truly disease-modifying and safer agents or dietary supplements for the management of OA are still needed.

The chondroprotective effects of sesamin were previously reported [11]. The present study has revealed for the first time that sesamin increases the HAC anabolism *via* elevation of aggrecan synthesis as demonstrated by the elevation of the gene expression of *ACAN*, *XYLT1*, *XYLT2*, *CHSY1*, and *CHPF*. The time-course study of the monolayer culture of HAC revealed that the gene expression of all tested CS biosynthetic enzyme genes peaked at 45 min and 5–6 h of sesamin treatments. This was concomitant with a previous report that the expression of *ACAN* was up-regulated at 5 h after mechanical stimulation [36]. In contrast, the pellet culture of HAC showed that the gene expression of all tested CS biosynthetic enzymes gradually increased and peaked at day 14 of sesamin treatments. Shi *et al.* have reported that multiple growth factors stimulated the expression of *ACAN* in articular chondrocytes. The *ACAN* expression peaked at day 3 after treatment with bone morphogenetic protein (BMP)-2 or BMP-7 and at day 5 with insulin-like growth factor (IGF)-1, IGF-2 or transforming growth factor-β1 [37]. These observations indicate that the timing of the up-regulation of *ACAN* can differ when stimulated with different growth factors or with certain plant extracts. Since our results suggested that the effects of sesamin are comparable to those of growth factors, it will be important to investigate if the effects of sesamin are exhibited by activating signaling pathways associated with growth factors.

To enable an accurate comparison of the quantity of GAGs secreted into the culture media or accumulated in the pellet

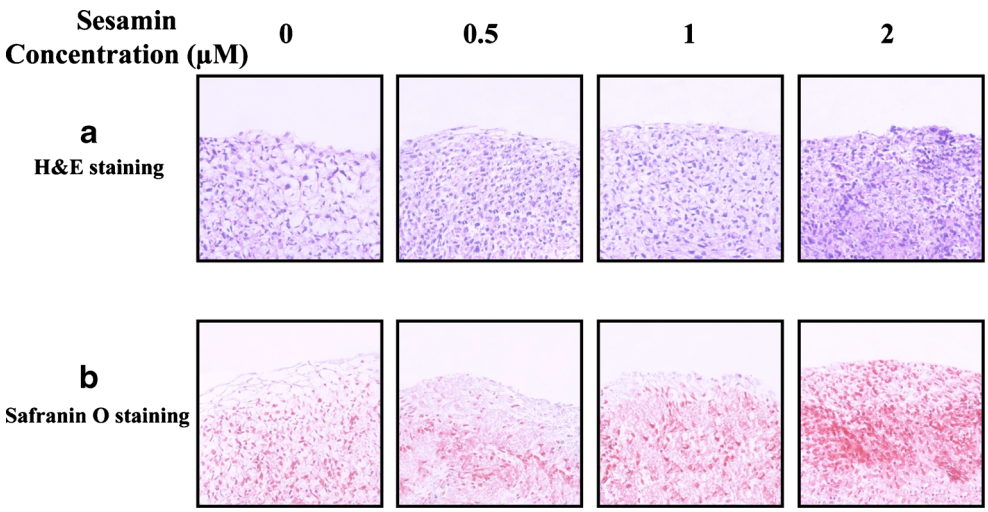

Fig. 6 The effects of sesamin on the accumulation of GAGs in the matrix. H&E staining (**a**) and Safranin-O staining (**b**) of HAC pellet sections on day 21. Sections (observed at 400× magnification) of HAC pellets treated with 0, 0.5, 1 and 2 μM sesamin are shown (from *left to right*)

matrix, the GAG content was normalized to the DNA content. However, in the monolayer culture, the GAG level in the culture media was not normalized because the culture media were collected at 45 min, and this time point was too short for the cells to proliferate since the doubling time of human cells for one cell cycle is approximately 24 h [38].

Two different culture methods were used in this study; monolayer and pellet cultures to investigate the effects of sesamin. The monolayer culture has some advantages for cell expansion and in measuring the effects of sesamin on the gene expression involved in CS-PGs biosynthesis for a short time period (within 24 h). The isolated HAC of late passages in monolayer culture will de-differentiate, in this environment, chondrocytes gradually lose their spherical shape and acquire an elongated fibroblast-like morphology. These morphological alterations are accompanied by profound biochemical changes such as the reduction or total loss of the synthesis of aggrecan and type II collagen (cartilage-specific proteins), and by the increase in the synthesis of versican and type I collagen. De-differentiated chondrocytes at low passage (<5) have the capacity to re-differentiate when transferred into an environment supporting a spherical morphology like pellet culture conditions, in which the synthesis of aggrecan and type II collagen are recovered [29]. Thus, the pellet culture method was used as a model to investigate the effects of sesamin on HAC with a cell morphology similar to that of chondrocytes in cartilage. The pellet culture was used also to confirm the accumulation of the extracellular matrix by histology.

In the present study, the expression of the genes of the biosynthesis of CS-PGs in HAC was activated by sesamin, which was concomitant with an increase in the up-regulated CS-PG level of the media of both the monolayer and pellet cultures. The rate of biosynthesis of CS-GAGs in chondrocytes is entirely dependent on the supply of the core protein of aggrecan PG encoded by *ACAN*. The biosynthesis of CS-GAGs is controlled by glycosyltransferases including XYLT, CHSY1, and CHPF [39–41]. This was consistent with our previous report that increasing the expression of the transcription factor SOX9 in HAC resulted in an increase in the GAGs biosynthesis in monolayer and pellet cultures [39]. These observations indicate that the effects of sesamin on the expression of the genes for the CS-PG biosynthesis may be associated with direct effect on SOX9. It will be important to investigate the regulation of the expression of the genes encoding CS-PG core proteins and the enzymes for CS biosynthesis through the SOX9 signaling pathway. Furthermore, it would also be interesting to extend the present study by investigating if sesamin can reverse any of the catabolic effects of inflammatory cytokines on HAC or can reverse changes in the gene expression in HAC isolated from OA patients. This will add to the present study, which examined the effects of sesamin on normal primary HAC from healthy joints.

In conclusion, the present study showed that sesamin had important anabolic effects on aggrecan production by primary cultures of HACs. Sesamin was shown to up-regulate the expression of the genes involved in the biosynthesis of CSPGs: *ACAN*, *XYLT1*, *XYLT2*, *CHSY1*, and *CHPF*. These effects were consistent with the increased secretion of GAGs into the media of the monolayer cultures as well as in 3D pellet cultures. These findings give insights into a possible treatment for OA using sesamin and may form the basis for the new therapeutic strategy using phytochemicals.

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Author contribution All authors have participated in the work as following:

PP—drafting of the article, interpretation of the data, final approval of the article, conception and design of the study.

SN—analysis and interpretation of the data, drafting the article, statically analysis.

JS—Histological analysis and interpretation.

SM—drafting of the article, final approval of the article.

KS—final approval of the article, revising for intellectual content, conception and design of the study.

PK—final approval of the article, revising for intellectual content, conception and design of the study.

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