

Inhibition of hyaluronan synthesis alters sulfated glycosaminoglycans deposition during chondrogenic differentiation in ATDC5 cells

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Abstract In chondrogenic differentiation, expression and collaboration of specific molecules, such as aggrecan and type II collagen, in extracellular matrix (ECM) are crucial. However, few studies have clarified the roles of hyaluronan (HA) in proteoglycan aggregation during chondrogenic differentiation. We assessed the roles of HA in sulfated glycosaminoglycans deposition during chondrogenic differentiation by means of 4-methylumbelliferone (4-MU), an HA synthase inhibitor, using ATDC5 cells. ATDC5 cells were treated with 0.5 mM 4-MU for 7 or 21 days after induction of chondrogenic differentiation with insulin. Depositions of sulfated glycosaminoglycans were evaluated with Alcian blue staining. mRNA expression of ECM molecules was determined using real-time RT-PCR. The deposition of aggrecan and versican was investigated with immunohistochemical staining using specific antibodies. Effects of 4-MU on HA concentrations were analyzed by HA binding assay. 4-MU suppressed the positivity of Alcian blue staining, although this delay was reversible. Interestingly, stronger positivity of Alcian blue staining was observed at day 21 in cultures with 4-MU discontinuation than in the control. 4-MU significantly increased the mRNA

expression of aggrecan, versican, and type II collagen, which was consistent with increased deposition of aggrecan and versican. The HA concentration in ECM and cell-associated region was significantly suppressed with 4-MU treatment. We conclude that the inhibition of HA synthesis slows sulfated glycosaminoglycans deposition during chondrogenic differentiation despite the increased deposition of other ECM molecules. Transient starvation of HA with 4-MU accelerates chondrogenic ECM formation, suggesting its potential to stimulate chondrogenic differentiation with adequate use.

Keywords Chondrogenesis · Hyaluronan · ATDC5 · Extracellular matrix

Introduction

Hyaluronan (HA), a linear glycosaminoglycan composed of repeated disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, is distributed widely in the extracellular space of connective tissues. HA plays prominent roles in regulating cell migration and proliferation in various dynamic processes such as tumorigenesis, wound-healing, and embryonic development (Laurent and Fraser 1992; Toole 1997).

Of the diverse roles of HA, it serves as a major component of the extracellular matrix (ECM) in cartilage and is critical for its structural organization and function. HA synthesized mainly by HA synthase-2 (Nishida et al. 1999) forms the backbone of the large proteoglycan aggregates in cartilage (Hascall and Heinegard 1974) and retains the highly charged sulfated glycosaminoglycans within the cartilage matrix. The proteoglycan aggregates interact with type II collagen. As a result, the viscoelastic property of

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the collagen fiber network and the osmotic pressure of proteoglycan facilitate the load-bearing functions of cartilage (Hardingham and Fosang 1992).

HA has crucial roles other than those in mature articular cartilage. During skeletal development, chondrogenesis is the process beginning with mesenchymal cell recruitment and migration, proliferation and condensation that results in the formation of the cartilage, and leads to endochondral ossification (Goldring et al. 2006). Previous reports suggested the role of HA and the interaction between HA and CD44, a major HA receptor, during chondrogenic differentiation. Mesodermal cells of limb bud from different development stages show that, at the time of cellular condensation in the limb bud, removal of HA by HAases is essential for the initiation of differentiation (Frost et al. 1997; Knudson and Toole 1985). In avian limb models, blocking HA binding to cell surface receptor using HA hexasaccharides prevents matrix assembly and retention, thereby inhibiting *in vitro* chondrogenesis (Maleski and Knudson 1996). However, few studies have clarified the role of HA by inhibition of HA synthesis in deposition of sulfated glycosaminoglycans during chondrogenic differentiation.

In a previous study, the accumulation of HA in the cell layer was substantially accelerated during the hypertrophic stage in cultured growth plate chondrocytes, and the expression of hyaluronan synthase (HAS) 2 mRNA was upregulated after the matrix-forming stage, with the expression level of HAS2 mRNA becoming maximal at the pre-hypertrophic stage (Suzuki et al. 2005).

4-Methylumbelliferone has been reported to inhibit HA synthesis dose-dependently in several cell types (Kakizaki et al. 2004; Kultti et al. 2009). Although the mechanism of 4-MU underlying the inhibition of HA synthesis has not been completely elucidated, a recent report showed that 4-MU inhibited HA synthesis by depletion of cellular UDP-glucuronic acid and downregulation of HAS2 and/or HAS3 in tumor cells (Kultti et al. 2009). This agent facilitates the analysis of the effects of HA inhibition on chondrogenic differentiation, particularly deposition of sulfated glycosaminoglycans. Moreover, it remains unknown whether the inhibition of HA influences other ECM molecules, such as aggrecan, type II collagen, and versican in chondrogenesis.

ATDC5 cells could replicate the multistep chondrogenic differentiation process during endochondral bone formation (Atsumi et al. 1990; Shukunami et al. 1996). In a previous study, treatment with 4-MU reduced sulfated glycosaminoglycans content and chondrogenic nodule formation by suppression of UDP-glucose dehydrogenase (UGDH) expression in chick limb bud micromass cultures (Clarkin et al. 2011). However, our preliminary experiment demonstrated that treatment with 4-MU did not affect UGDH mRNA expression in ATDC5 cells (see supplemental Fig),

indicating 4-MU would be an appropriate agent that inhibits HA specifically in this ATDC5 model. We hypothesized that inhibition of HA synthesis with 4-MU treatment would alter the levels of sulfated glycosaminoglycans during chondrogenic differentiation in ATDC5 cells and reveal the roles of HA in chondrogenesis.

The purpose of the present study was to clarify the role of HA in sulfated glycosaminoglycans during chondrogenic differentiation using ATDC5, a mouse chondrogenic cell line, by inhibition of HA.

Materials and methods

Cell culture

A mouse chondrogenic cell line, ATDC5, was purchased from the RIKEN Cell Bank (Tsukuba Science City, Japan). ATDC5 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Sigma, ST. Louis, MO, USA) containing 5 % fetal bovine serum and antibiotic (penicillin: 100 U/ml, streptomycin: 100 µg/ml, amphotericin-B: 0.25 µg/ml) at 37 °C under 5 % CO₂ in air. ATDC5 cells were seeded at a density of 1×10^5 cells/well in 6-well plates. To induce chondrogenic differentiation, ITS Liquid Media Supplement (Sigma) was added to the medium at a concentration of 10 µg/ml after confluence. ATDC5 cells were cultured for 21 days (confluent on day 0). The medium was replaced once every 2 days. 4-MU stock solution for experiments was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in medium was adjusted to 0.1 %. All incubations were performed in the presence of 5 % FBS.

Treatment with 4-MU and/or HA

ATDC5 cells were seeded at a density of 1×10^5 cells/well onto 6-well plates. After confluence, the cells were cultured in the medium supplemented with ITS for 21 days (day 0 to day 21). As a prerequisite for the experiments, ATDC5 cells were treated with various concentrations (0.1, 0.5, 1.0, 2.0 mM) of 4-MU. HA concentration in the pericellular region was measured with HA binding assay. The preliminary results showed that 0.5 mM of 4-MU effectively inhibited HA, and so 4-MU was used at 0.5 mM concentration for all subsequent experiments. From day 0, the cells were treated every 2 days with 4-MU (0.5 mM) for 7 or 21 days. To analyze whether exogenous HA is able to cancel the effects of 4-MU, cell proliferation was determined by co-incubation of 0.5 mM 4-MU with or without 1 mg/ml HA (Julovi et al. 2004) of exogenously added HA (Artz; molecular weight 600–1200 kDa) (Seikagaku, Tokyo, Japan) for 21 days. Artz was used as an exogenous HA because the

molecular weight (600–1200 kDa) is within ranges (300–2000 kDa) of HAS2 products. HAS2 has been reported to be a main HAS in articular cartilage and chondrogenesis (Nishida et al. 1999). In addition, solitary effects of exogenous HA (1 mg/ml) were also investigated. 4-MU was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in the medium was adjusted to 0.1 %. As a control, the cells were treated with 0.1 % DMSO.

Alcian blue staining

To evaluate the deposition of sulfated glycosaminoglycans during chondrogenic differentiation, proteoglycan-rich matrix was stained with Alcian blue. Cells were fixed with 100 % methanol and stained with 0.1 % Alcian blue 8GS (Sigma) in 0.1 N HCl for 4 h at room temperature. Deposition of proteoglycan-rich matrix was determined with this staining every 7 days (day 0, day 7, day 14, and day 21). For quantitative analyses of Alcian blue-positive area, the dye was extracted with 6 M guanidine HCl overnight, and the total optical density of the extracted dye was measured using a spectrophotometer at 620 nm.

Real-time RT-PCR

Expression levels of aggrecan, type II collagen, versican, HAS2, and CD44 mRNAs were determined with real-time RT-PCR. Total RNAs were isolated from cultured ATDC5 cells (every 3 days from day 0 to day 21) using RNeasy® Mini Kit (QIAGEN, Tokyo, Japan), and reverse-transcribed to cDNA using high-capacity cDNA reverse transcription kit (Applied Biosystems, CA, USA).

Reverse-transcribed cDNA was subjected to real-time PCR using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with a LightCycler 480 SYBR Green I kit (Roche Molecular Biochemical, Mannheim, Germany). The relative levels of target mRNAs in a sample were expressed as relative quantification normalized against GAPDH mRNA levels. The primer sequences of the aggrecan, versican, type II collagen, HAS2, CD44, and GAPDH used were as follows: for aggrecan, 5'-ATGAGAGAGGCG AATGGAAC-3' (forward) and 5'-ACCAGGGAGCTGATC TCGTA-3' (reverse); for versican, 5'-CCAGTGTGAACCTT GATTTTGATGAA-3' (forward) and 5'-AACATAACTTG GGAGACAGAGACATCT-3' (reverse); for type II collagen, 5'-CCAGGGCTCCAATGATGTAG-3' (forward) and 5'-CGGTACTCGATGACGGTCT-3' (reverse); for HAS2, 5'-CGTACATCTGGAAGAACAACCTT-3' (forward) and 5'-ACCTGTACATAATCCACGCTT-3' (reverse); for CD44, 5'-TGGCTCATCATCTTGGCATCT-3' (forward) and 5'-GATGCAGACGGCAAGAATCA-3' (reverse); and for GAPDH, 5'-CACTCTTCCACCTTCGATGC-3' (forward) and 5'-TCTTGCTCAGTGTCTTGGCT-3' (reverse).

Immunocytochemistry for HA-binding proteoglycans and HA staining

The cells were seeded at a density of 1×10^4 cells/well onto chamber slides (BD Biosciences) and cultured in the medium supplemented with ITS. The cells were treated with or without 0.5 mM 4-MU for 7 or 21 days. The cells were fixed at days 0, 7, 14, and 21 with 4 % paraformaldehyde buffered with phosphate-buffered saline (PBS) at room temperature for 2 h and subjected to immunocytochemical analysis using the specific antibodies or 2.0 µg/ml of a biotinylated HA-binding protein (HABP) (Seikagaku, Tokyo, Japan) at room temperature for 2 h. Primary antibodies used were anti-mouse aggrecan polyclonal antibody (1:100 dilution) (Millipore, Billerica, MA, USA) and anti-mouse versican polyclonal antibody (1:100 dilution) (Millipore, Billerica, MA, USA). Immunofluorescence staining for HA and aggrecan was performed in cultured cells treated with or without 0.5 mM 4-MU at day 7. Each image was merged to determine the colocalization of HA and aggrecan.

HA binding assay

After induction of chondrogenic differentiation with ITS, ATDC5 cells were treated with or without 0.5 mM 4-MU. HA concentrations in medium and pericellular region were measured every 3 days for 20 days according to a protocol described previously (Zhu et al. 2010). Briefly, the conditioned medium was collected and designate as the “medium” pool. To remove the cell-surface-associated HA, the cells were incubated for 10 min at 37 °C with trypsin–EDTA and washed with PBS. The trypsin solution and combined washes were designated as the “extracellular and cell-associated” pool. All samples were heated at 100 °C for 15 min to inactivate protease activity, centrifuged at 15,000g for 30 min at 4 °C, and then the supernatants were subjected to HA binding assay to measure the HA concentrations (Zhu et al. 2010).

Statistical analysis

All of the quantitative experiments were performed more than three times. Data are expressed as the mean \pm SEM. To access differences between means, the Bonferroni/Dunn test and the nonparametric Mann–Whitney *U* test were used. *P* values of <0.05 were considered significant.

Results

Differentiation process of ATDC5

ATDC5 cells have properties to reproduce multisteps of chondrogenic differentiation synthesizing proteoglycan

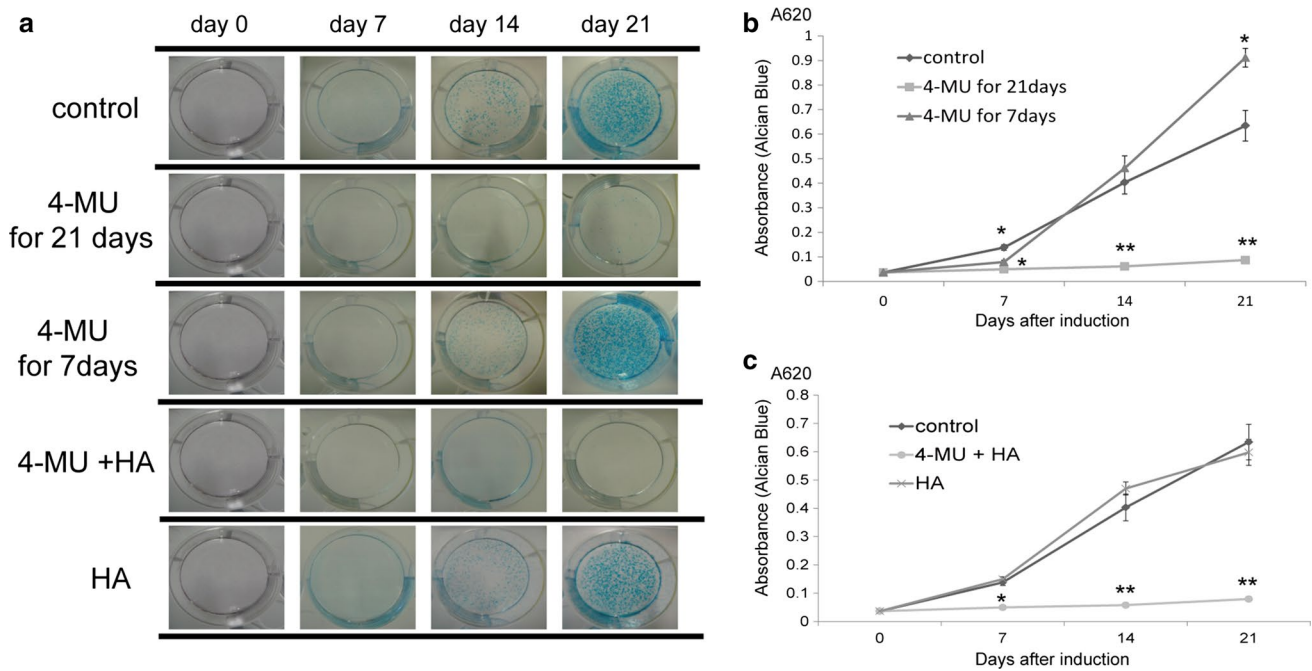


Fig. 1 Effect of 4-MU on cartilage nodule formation and Alcian blue stainable production of proteoglycan in ATDC5 cells. **a** ATDC5 cells were cultured in 6-well plates in the presence of ITS Liquid Media Supplement with 0 or 0.5 mM of 4-MU for 21 or 7 days. HA (1 mg/ml) was added with or without 0.5 mM of 4-MU in other cultures. Cultured wells were photographed at days 0, 7, 14, and 21. **b** The quantitative measurement of proteoglycan production. Cultures

without or with 0.5 mg of 4-MU for 7 or 21 days were subjected to quantification at days 0, 7, 14, and 21. The total optical density of dye extracted with guanidine HCl was measured using a spectrophotometer at 620 nm. **c** Cultures with or without 1 mg/ml of HA and/or 0.5 mM of 4-MU were subjected to quantification at days 0, 7, 14, and 21. Error bars express \pm SEM. * $p < 0.05$ or ** $p < 0.01$ depicted treatment group versus control group by Bonferroni/Dunn analysis

and type II collagen when treated with ITS. Without 4-MU treatment, condensation of committed cells to form nodules occurred day 7 to day 21. Marked proteoglycan deposition, which is characteristic of ECM in cartilage, was observed at day 14 and day 21 with Alcian blue staining (Fig. 1). Dynamic chondrogenic markers (mRNAs of aggrecan and type II collagen) peaked at days 12–15 (Fig. 2), whereas the hypertrophic marker (mRNA of type X collagen) peaked at day 21. These results validated the use of ATDC5 cells as an in vitro model of chondrogenic differentiation in the subsequent investigation. In this model, ATDC5 cells undergo chondrogenic differentiation at around days 12–15 and undergo hypertrophic differentiation at around day 21.

Altered differentiation process of ATDC5 with 4-MU

We examined the effects of inhibition of HA synthesis using 4-MU on deposition of proteoglycan and cartilage nodule formation. Control culture of ATDC5 cells underwent predictable chondrogenic–hypertrophic differentiation in insulin-containing medium, as observed by increased Alcian blue staining at day 14 for cartilaginous ECM (Fig. 1), and Alizarin Red S staining for mineralized matrix, while the cultures treated with 4-MU showed markedly decreased positive staining with Alcian blue (Fig. 1)

and no staining of subsequent mineralized ECM. A quantitative determination for Alcian blue-positive proteoglycan deposition showed that the 4-MU treatment resulted in the complete suppression of Alcian blue positivity.

It would be important to know whether cells treated with 4-MU, which do not show Alcian blue-positive staining, are still progenitor-like cells or mature chondrogenic cells without the capacity for the formation of proteoglycan aggregates in ECM. The cells were treated with 4-MU for 21 days, followed by the discontinuation of 4-MU for 2 days in insulin-containing medium, and stained with Alcian blue. The positivity of Alcian blue staining in this culture was markedly fainter than that in control culture at day 21. The positivity was approximately equal to that in control cells at 7 days. This result indicates that ATDC5 cells treated with 4-MU at day 21 fail to differentiate into chondrocyte with the capacity for the formation of proteoglycan aggregate in ECM.

Recovery from 4-MU-inhibited deposition of sulfated glycosaminoglycans

The reversibility of the inhibitory actions of 4-MU was examined in experiments in which 4-MU was removed at day 7. Following the addition of control differentiation medium with discontinuation of 4-MU treatment, the cells

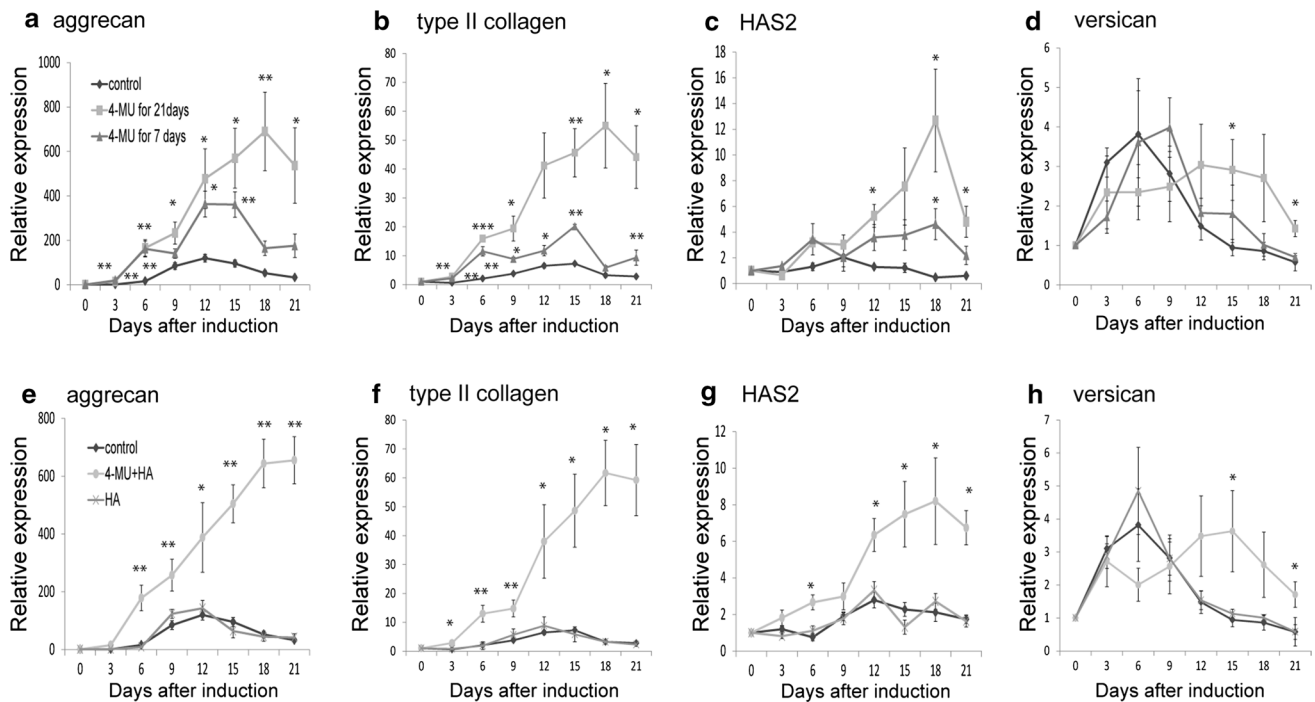


Fig. 2 Effect of 4-MU on chondrogenesis-related gene expression. ATDC5 cells were cultured with or without 0.5 mM of 4-MU for up to 7 or 21 days. On days 0, 3, 6, 9, 12, 15, 18, and 21 of culture, expression of aggrecan (**a**), type II collagen (**b**), HAS2 (**c**), and versican (**d**) mRNA was determined using real-time reverse-transcribed polymerase chain reaction (RT-PCR). ATDC5 cells were also cultured without or with 1 mg/ml of exogenous HA \pm 4-MU. On days 0, 3, 6, 9, 12, 15, 18, and 21 of culture, expression of aggrecan (**e**), type II collagen (**f**), HAS2 (**g**), versican (**h**) mRNA was determined. The

data presented are relative mRNA expression values standardized by GAPDH mRNA expression. 4-MU significantly increased the expression of aggrecan, type II collagen, and versican during chondrogenic differentiation. On the other hand, exogenous added HA did not cancel out the effect of 4-MU on mRNA expression of matrix molecules. Error bars express \pm SEM. * $p < 0.05$ or ** $p < 0.01$ or *** $p < 0.001$ depicted treatment group versus control group with Bonferroni/Dunn analysis

reentered standard process of chondrogenesis with marked positive staining with Alcian blue. Interestingly, stronger positivity of Alcian blue staining was observed at day 21 in cultures with 4-MU discontinuation than in the control (Fig. 1a, b), suggesting that a transient starvation state of HA with 4-MU may stimulate sulfated proteoglycans deposition in non-damaged cells. Similar results were obtained from experiments in which 4-MU was removed at day 14.

Effects of exogenous added HA in cultures treated with 4-MU

Given that intracellular HA (internalized HA) and/or HA synthase-bound HA may be functionally different from extracellular HA, we examined whether inhibitory effects of 4-MU for the deposition of sulfated glycosaminoglycans during chondrogenic differentiation could be cancelled with addition of exogenous HA. Co-treatment of exogenous HA (1 mg/ml) with 4-MU did not cancel the inhibitory effects of 4-MU (Fig. 1a, c), indicating that 4-MU inhibition of HA synthesis cannot be compensated for with

exogenous HA, and so HA inhibited by 4-MU is likely to be functionally different from exogenously added HA.

The effect of 4-MU on chondrogenesis-related gene expression

The effect of 4-MU on the deposition of proteoglycan aggregates during chondrogenic differentiation at the molecular level was further examined by quantitating the expression levels of the chondrocyte and chondrogenic differentiation marker gene: aggrecan, versican, type II collagen, in addition to HAS2, which dominantly synthesizes HA in articular cartilage (Nishida et al. 1999), and CD44, which is the main cell surface receptor for HA. As a result, the mRNA expression of aggrecan significantly increased in the cells treated with 4-MU compared with control cells from day 6 to the end of treatment (day 21) (Fig. 2a). Similar results were obtained with type II collagen (Fig. 2b) and HAS2 (Fig. 2c). These results indicated that 4-MU had no inhibitory effects on mRNA expressions of chondrogenic ECM molecules; however, abrogation of proteoglycan-rich ECM formation detected with Alcian blue

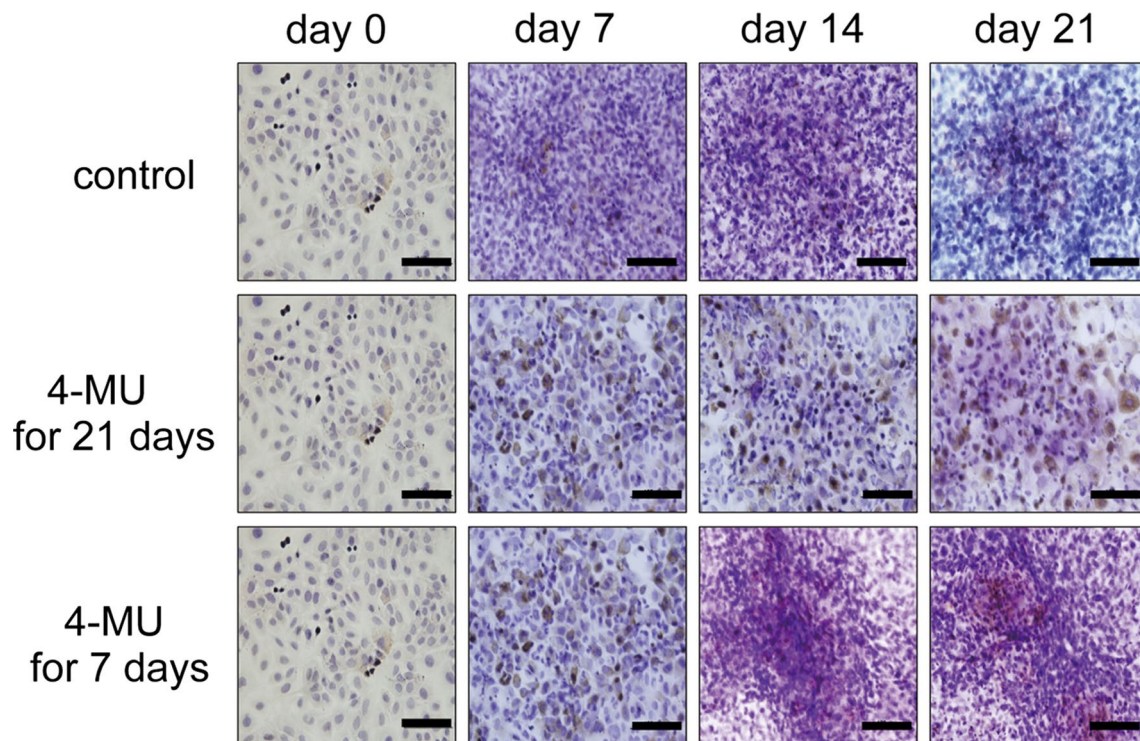


Fig. 3 Immunocytochemical of aggrecan in ATDC5 cells. ATDC5 cells were cultured with or without 0.5 mM of 4-MU for up to 7 or 21 days. On days 0, 7, 14, and 21, cultures were subjected to immunohistochemical analysis for aggrecan. Bar 100 μ m

staining (Fig. 1) may feedback positively to mRNA expression of aggrecan, type II collagen, and HAS2. Versican is a large chondroitin sulfate proteoglycan of ECM with a domain structure common to that of aggrecan (Shinomura et al. 1993). In developing cartilage, versican is transiently expressed at a high level in the mesenchymal condensation area and rapidly disappears during cartilage development (Kimata et al. 1986). To evaluate the stage prior to chondrogenic differentiation, versican can be a good marker molecule. As expected, versican mRNA expression peaked at day 6 and decreased immediately prior to chondrogenic differentiation. 4-MU treatment maintained mRNA expression of versican at high levels, suggesting that the cells under 4-MU will maintain the pre-chondrogenic phenotype (Fig. 2d). Intriguingly, exogenously added HA did not cancel the effects of 4-MU on mRNA expressions of aggrecan, type II collagen, HAS2, and versican, nor have stimulatory effects on chondrogenic differentiation with solitary use (Fig. 2e–h). The mRNA expression of CD44 was not affected by 4-MU treatment.

Immunocytochemical localization of aggrecan, versican, and HA during chondrocyte differentiation

Immunocytochemical staining for aggrecan was detected on areas of cell condensation at day 7, and the positivity of staining weakened thereafter in the control culture. On the

other hand, 4-MU markedly increased the positive staining for aggrecan until day 21 (Fig. 3). Together with the findings of Alcian blue and mRNA expressions, although 4-MU stimulated the mRNA expression of cartilage matrix molecules, the integrity of ECM formation could not be maintained under suppression of HA synthesis.

The staining for versican increased in a stage prior to chondrogenic differentiation and then decreased rapidly in the control cultures. 4-MU prolonged the positive staining for versican to day 21 (Fig. 4).

The site of nodule formation in the control cultures showed an accumulation of HA at day 21. On the other hand, little HA positivity was seen in the cultures treated with 4-MU at day 21 (Fig. 5).

Results of immunofluorescence staining for HA and aggrecan (Fig. 6) showed that merged images of HA and aggrecan staining showed that HA and aggrecan colocalized in area of nodule formation in the control cultures at day 7. On the other hand, in cultures with 4-MU treatment, decreased accumulation of HA and increased aggrecan positivity were not colocalized at day 7.

Quantification of HA in cultures of ATDC5 cells

The concentration of “extracellular and cell-associated” HA treated with 4-MU was significantly lower than that

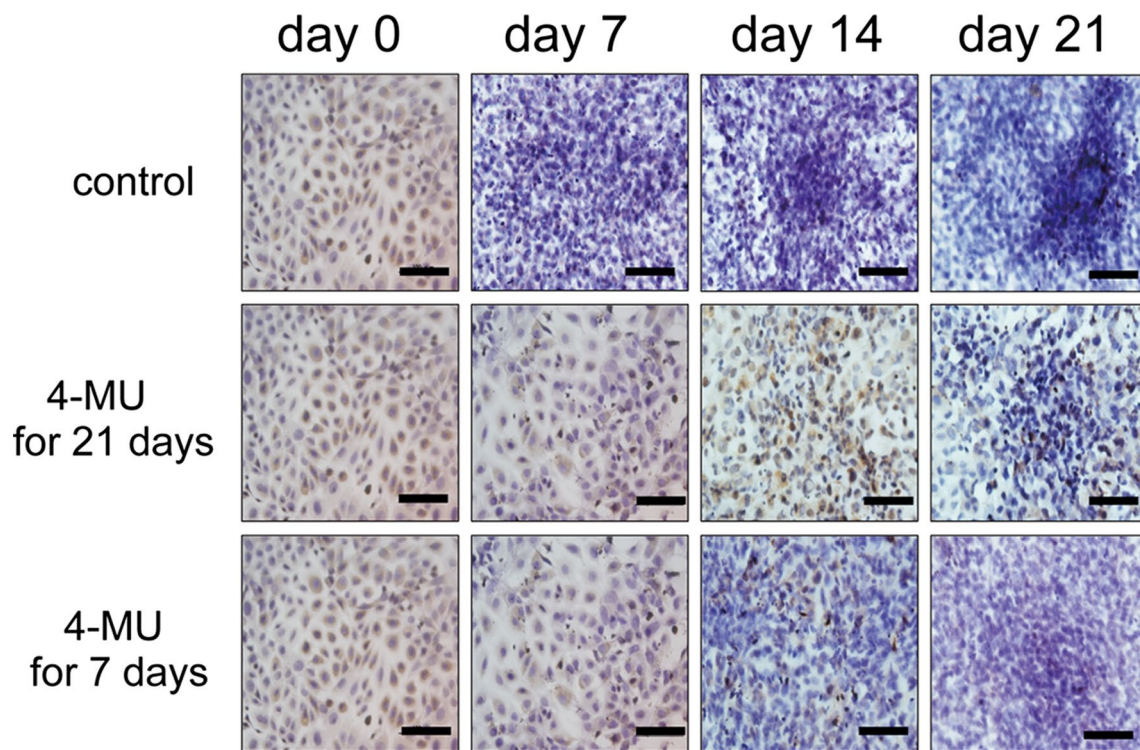


Fig. 4 Immunocytochemical of versican in ATDC5 cells. ATDC5 cells were cultured with or without 0.5 mM of 4-MU for up to 7 or 21 days. On days 0, 7, 14, and 21, cultures were subjected to immunohistochemical analysis for versican. Bar 100 μ m

of the control cells over the entire chondrogenic differentiation process (Fig. 7a). In contrast, the concentration of medium HA in cultures treated with 4-MU was not statistically different from that without 4-MU (Fig. 7b). These results indicated that “extracellular and cell-associated” HA, which might be a crucial molecule to form large proteoglycan aggregates (Nishida et al. 2000), is inhibited with 4-MU, resulting in the suppressed deposition of sulfated glycosaminoglycans.

Discussion

The mouse embryonal carcinoma-derived cell line ATDC5 provides a good model system to study chondrogenesis *in vitro* (Atsumi et al. 1990). In the long-term culture system, ATDC5 cells can trace all steps of chondrogenic differentiation from the pre-condensation stage to the calcified cartilage stage. Several previous studies investigated the roles of HA in the process of chondrogenesis. Knudson et al. reported that micromass cultures of chick limb bud with HA hexasaccharides (HA6) resulted in disruption between native macromolecules of HA and cell surface receptor binding, and decreased cellular condensation and matrix production, resulting in a temporal delay of chondrogenic differentiation of mesenchymal cells (Knudson et al. 1993;

Maleski and Knudson 1996). Although the suppression of native HA using HA6 in Knudson’s study or 4-MU in this study shares a similar concept, exogenously added HA6 may have different roles as a ligand of cell surface receptors, such as CD44. Clarkin et al. (2011) demonstrated that micromass cultures from chick limb bud treated with 4-MU suppressed HA markedly and sulfated glycosaminoglycans slightly, leading to decreased deposition of Alcian blue stainable matrix proteoglycan. However, their study did not investigate the time course of the inhibitory effects with 4-MU, nor whether alteration of proteoglycan deposition with 4-MU is reversible or not. The current study clarified the time course of mRNA expressions of ECM molecules, which was continuously upregulated under the influence of 4-MU, although Alcian blue stainable proteoglycans were decreased. This may be explained for the theory that inhibition of HA synthesis prevents retention of ECM, producing a positive feedback loop leading to the overexpressions of aggrecan, type II collagen.

One of the most noteworthy findings in the current study is that the delay in deposition of sulfated glycosaminoglycans during chondrogenic differentiation is reversible after discontinuation of 4-MU. Moreover, recovered cultures from HA suppression state had more abundant Alcian blue-positive proteoglycan than those of control, suggestive of a rebound phenomenon after discontinuation. These results

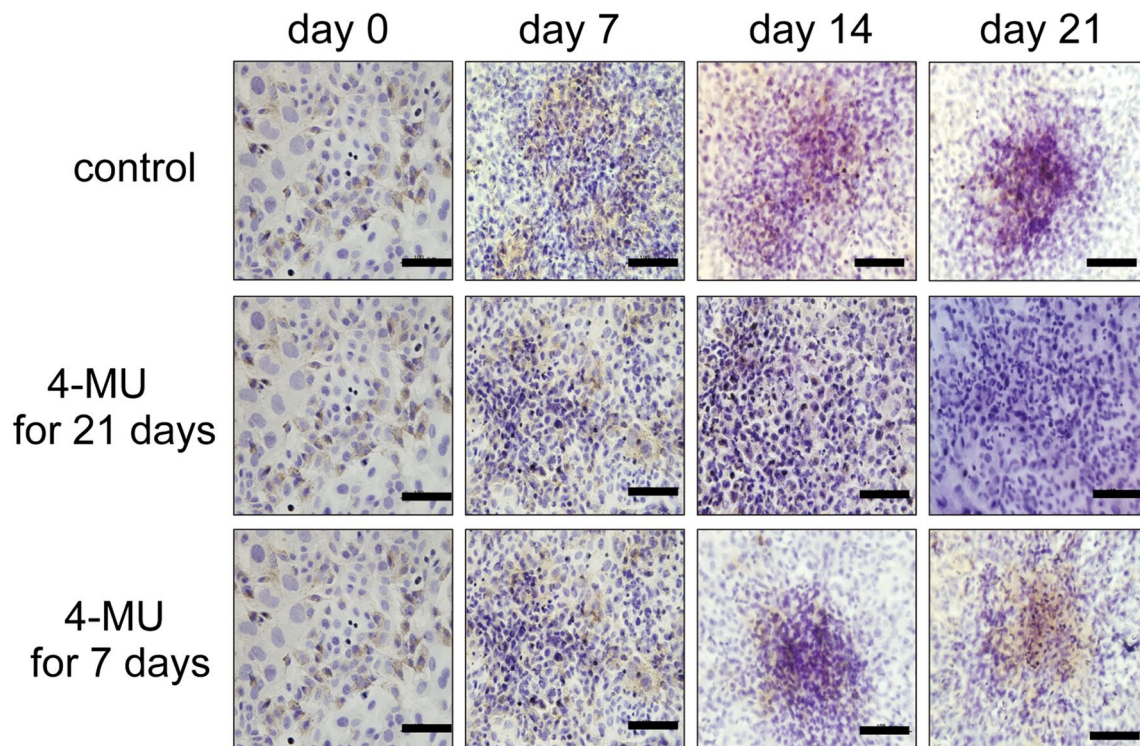


Fig. 5 Cytochemical staining of HA in ATDC5 cells. ATDC5 cells were cultured with or without 0.5 mM of 4-MU for up to 7 or 21 days. On days 0, 7, 14, and 21, cultures were subjected to histochemical analysis for HA. Bar 100 μ m

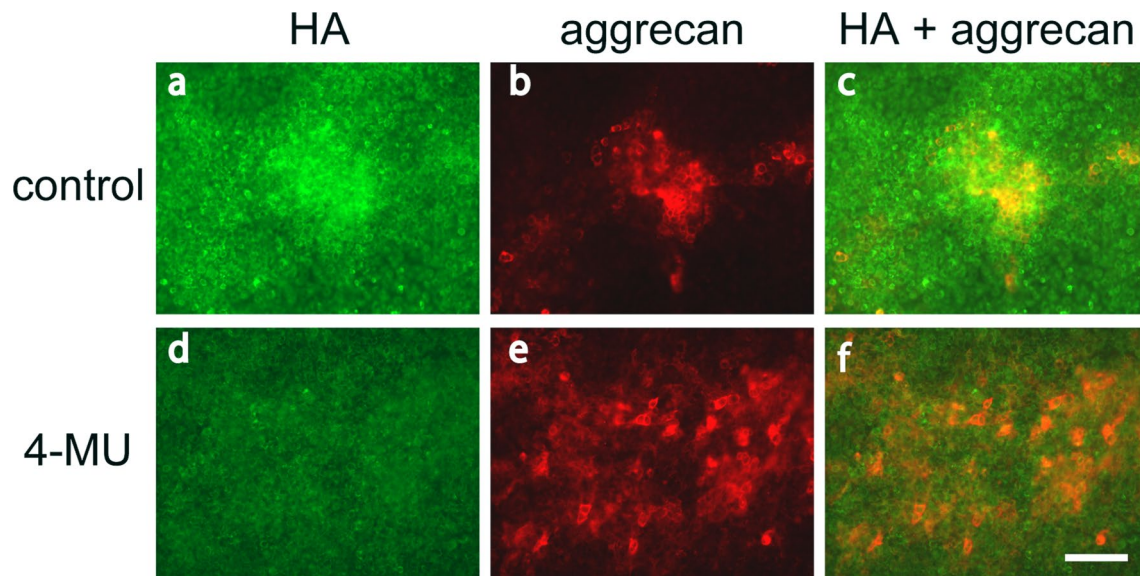


Fig. 6 Fluorescent double staining of HA and aggrecan. ATDC5 cells were cultured with (d, e, f) or without (a, b, c) 0.5 mM of 4-MU for 7 days, subjected to fluorescent staining for HA (a, d), aggrecan (b, e), and merged (c, f). Bar 100 μ m

hint at the possibility of stimulation with 4-MU for regenerative activity of chondrogenesis.

Inhibition of HA synthesis with 4-MU could not be cancelled by exogenously added HA, as previously reported in

studies of malignant neoplasms (Arai et al. 2011; Urakawa et al. 2012). Several possibilities may explain this. One is that native HA inhibited with 4-MU is functionally different from exogenously added HA. HA synthesized by HAS

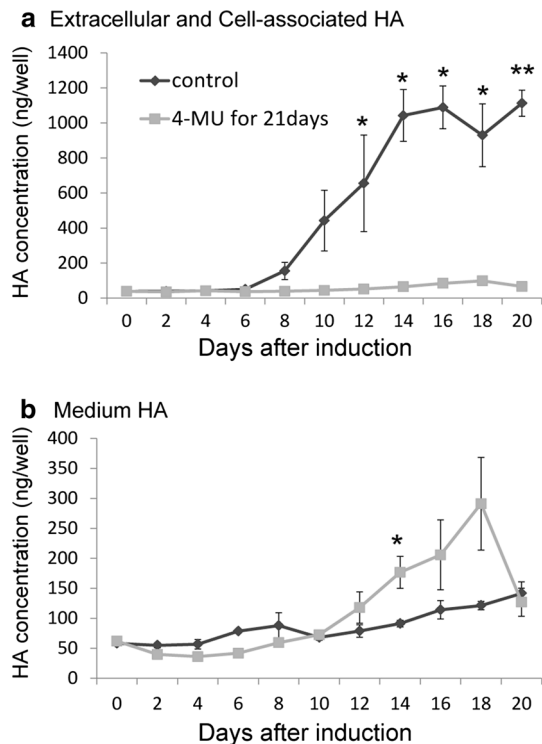


Fig. 7 Effects of 4-MU on HA concentrations in cultures. Medium and ECM or cell-associated matrix were collected and subjected to an HA binding assay. Error bars express \pm SEM. * $p < 0.05$ or ** $p < 0.01$ versus control group by Mann–Whitney U test

might be bound to cell-membrane HAS and/or to cell surface receptor such as CD44, whereas exogenous HA has a barrier to bind the precursor cells, which may inhibit the formation of cell-surface receptor-exogenous HA–aggrecan complex, and could not recover the Alcian blue staining. These differences may alter the intracellular signaling pathway or cell–cell and cell–matrix interactions (Knudson and Knudson 1993). Another possibility is that the higher concentration of exogenous HA than that used in this study may serve a function by replacement of the native HA bound to cell surface receptor (Nishida et al. 2004). However, while much higher concentrations of exogenous HA are convenient for experiments, they do not reflect the physiological condition of chondrogenesis.

In the bioengineering field, several previous studies have described the advantage of HA supplementation for matrix synthesis. Schwartz et al. (2011) demonstrated that high molecular weight HA supplementation in culture medium had a dose-dependent effect on matrix production during chondrogenic differentiation of MSCs on chitosan sponges. Yoshikawa et al. (2013) showed that 800 kDa of HA supplementation significantly increased aggrecan and type II collagen mRNA expression in ATDC5 cells on synthetic hydrogels. In the current study, exogenous HA had no effects of chondrogenic differentiation in ATDC5 cells,

which are exposed to more physiologic circumstances than bioengineering studies.

In developing cartilage, versican is transiently expressed at a high level in the mesenchymal condensation area and rapidly disappears during cartilage development (Kimata et al. 1986). Recent immunohistochemical studies on developing limb bud cartilage have revealed that an area positive for versican gradually shifts out of the diaphysis and is replaced by an area positive for aggrecan (Shibata et al. 2003). These reciprocal patterns of versican and aggrecan expression suggested that versican serves as a temporary framework in developing cartilage matrix. In this study, increased mRNA expression of versican was maintained by 4-MU treatment and also mRNA expression of aggrecan increased by 4-MU treatment, despite maintenance of increased expression of versican. These results may indicate that reciprocal expression of versican and aggrecan is abrogated in the absence of HA synthesis and deposition.

The mechanism of 4-MU underlying the selective inhibition of HA synthesis is known to be complex. A recent report using melanoma and carcinoma cells has suggested that 4-MU inhibits HA synthesis by depleting the cellular UDP-GlcUA pool (Kultti et al. 2009). However, such depletion of UDP-GlcUA in the cellular pool may be expected to affect the biosynthesis of other GlcUA-containing glycosaminoglycans, such as heparin and chondroitin sulfate. A possible explanation for the selective targeting of HA synthesis by 4-MU has been proposed. This includes relative insulation against changes in substrate availability in the Golgi versus the cytosol. Thus, the Golgi environment will be more controlled and less susceptible to large fluxes of substrates than the cytosol, due to the existence of its antiporter mechanism for substrate entry (Schwartz et al. 1998). These suggest the inhibitory effects of 4-MU in the current study may be specific for HA. The effects of 4-MU on HAS expression have been reported to be up- or downregulative in several cell types and have been thought to be cell type-dependent duration of treatment (Arai et al. 2011; Kultti et al. 2009; Urakawa et al. 2012). In this study, mRNA expression of the HAS2 was increased by 4-MU treatment. This result can be explained by the idea that the reduction of HA synthesis induced by UDP-GlcUA depletion by 4-MU causes positive feedback for HAS2 mRNA expression to compensate for the decreased deposition of HA. In our study, 4-MU had no significant effect on CD44 mRNA, a major cell surface receptor for HA. This result is in agreement with previous reports on other cell types, in which 4-MU did not alter CD44 mRNA (Arai et al. 2011; Kultti et al. 2009; Rilla et al. 2004).

There are some limitations in this study. Expression levels of aggrecan and versican mRNA were determined, while the amount of sulfated glycosaminoglycan was not precisely. There will be a discrepancy between expression

levels of mRNA and core protein expression and those of sulfated glycosaminoglycan. The effects of higher concentrations of exogenous HA should be analyzed further.

In conclusion, we demonstrated that treatment with 4-MU suppressed nodule formation and Alcian blue-positive staining despite the upregulation of aggrecan and type II collagen mRNA expression, suggesting that HA plays pivotal roles in retention of cartilage matrix during chondrogenic differentiation. Moreover, the delay in deposition of sulfated glycosaminoglycans during chondrogenesis with 4-MU is reversible, and starvation of HA by 4-MU stimulated the expression of proteoglycan and increased ECM formation as compared to control, suggesting that HA may be a target which can regulate functional glycosaminoglycan formation and alter cartilage metabolism.

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