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ORIGINAL ARTICLE

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Extracellular matrix recovery by human articular chondrocytes after treatment with hyaluronan hexasaccharides or *Streptomyces* hyaluronidase

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Abstract The treatment of human articular chondrocytes with *Streptomyces* hyaluronidase (*St*-HA′ase) or hyaluronan hexasaccharides (HA_6) provides two approaches to the selective depletion of specific components of the extracellular matrix, and an opportunity to follow the reparative responses initiated by these changes. In this study, changes in the relative expression of messenger RNA for hyaluronan synthase-2, CD44, and aggrecan were determined by competitive, quantitative reverse transcriptase–polymerase chain reaction. Changes in the size of the cell-associated matrix surrounding live chondrocytes were analyzed by the particle exclusion assay, and hyaluronan accumulation was characterized using a biotin-labeled hyaluronan-specific binding protein. Both *Streptomyces* hyaluronidase as well as hyaluronan hexasaccharide treatment of chondrocytes resulted in an approximately 2-fold increase in hyaluronan synthase-2 mRNA copy numbers, together with a 1.8-fold increase in the mRNA copy number for the proteoglycan aggrecan. However, although matrix biosynthesis was enhanced, the chondrocytes failed to retain these components. Both treatments resulted in a diminished accumulation of extracellular hyaluronan as well as a loss of the chondrocyte proteoglycan-rich cell-associated matrix. Thus, this model is similar to the early stages of osteoarthritis. Upon removal of the *Streptomyces* hyaluronidase or hyaluronan hexasaccharides, the normal, healthy, adult human chondrocytes used in this study regained their capacity to retain extracellular hyaluronan and to reassemble and retain a cell-associated matrix. This stimulation of hyaluronan synthase-2 (HAS-2) and aggrecan mRNA expression, and the subsequent capacity to retain the newly synthesized extracellular matrix, illustrate the events which are necessary for adult human articular chondrocytes to undergo effective repair.

Key words Articular chondrocytes · Extracellular matrix · Hyaluronan hexasaccharides (HA_6) · Hyaluronan synthase (HAS) · *Streptomyces* hyaluronidase (*St*-HA′ase)

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Introduction

An attempted but failed repair of the extracellular matrix of human articular cartilage is one suggested mechanism for the onset of osteoarthritis.[1] Of all the extracellular macromolecules present within cartilage, loss of proteoglycan represents one of the most common molecular symptoms associated with animal models and with naturally occurring human osteoarthritis. The loss of proteoglycan staining represents one of the parameters of the Mankin ranking system. The major proteoglycan of cartilage, aggrecan, is retained within cartilage as an aggregate with more than 50 aggrecan monomers bound to a single filament of another glycosaminoglycan, hyaluronan (HA).[2,3] Hyaluronan further facilitates the retention or anchorage of aggrecan within the tissue by the association of hyaluronan/aggrecan aggregates with the cell surface hyaluronan receptor CD44.[4,5] Appropriate expression levels of all three components, namely CD44, hyaluronan, and aggrecan, are necessary for the retention of aggrecan in the cell-associated matrix.

Recently, it has been emphasized that the rate of aggrecan synthesis is dependent on the age of the specimen from which the tissue was obtained.[6] The ability of the chondrocyte to make this molecule is not sufficient to form the proteoglycan aggregates in the extracellular matrix. The aggregation of matrix molecules would be important for normal tissue homeostasis, especially during the pathological process when the chondrocyte attempts to repair a degenerated extracellular matrix. We have previously reported results which suggest that optimal repair of extracellular matrix requires the coordinated biosynthesis of HA in

addition to aggrecan, as well as the expression of the HA receptor CD44.[4,5] If either CD44 or HA is not present, matrix assembly does not occur. In organ cultures of cartilage tissue slices, antisense inhibition of CD44 leads to loss of matrix retention,[7] and antisense oligonucleotides of hyaluronan synthase-2 (HAS-2), which is the predominant enzyme for HA synthesis in human articular chondrocytes, inhibits the cell-associated matrix assembly.[8] These results suggest that not only aggrecan production, but also biosynthesis of the components required for matrix retention and assembly are important in cartilage repair.

Pericellular HA was removed by either *Streptomyces* hyaluronidase (*St*-HA′ase) or hyaluronan hexasaccharides (HA_6), resulting in the depletion of the cell-associated matrix assembly in articular chondrocytes.[5,9,10] Thesefore, both represent relevant models for the induction of matrix depletion and for analyzing the ability of chondrocytes to recover the matrix assembly by articular chondrocytes. There are several other models of cartilage damage or recovery. Some employ trypsin, stromelysin, and MMPs.[11–13] Others use cytokines such as IL-1.[14] The problem with these approaches is that they lack specificity. As well as degradation of the extracellular matrix by enzymes such as trypsin, cell surface receptors, bound growth factors, etc., are also likely to be damaged. The use of *St*-HA′ase, on the other hand, offers a high degree of specificity.[4] Only extracellular HA is degraded. Thus, hyaluronidase provides a means to probe changes in cell-matrix interactions that depend upon only one matrix macromolecule. Hyaluronidase, which degrades both CD44-bound HA and synthase-bound HA, cannot distinguish whether reparative responses mounted by the chondrocytes are due to a loss of the HA-rich cell-associated matrix itself, or a response mediated via decreased occupancy of a matrix receptor. The use of HA_6 allows such a differential comparison to be made, as it only releases HA and PG bound via CD44. Neither HA attached to synthase nor PG bound to HA are released by HA hexasaccharides.[5,10] Putting these two together provides a unique approach to investigating the ability of chondrocytes to respond specifically to a carefully controlled loss of cell-associated matrix aggrecan.

In this study, the effects of matrix–chondrocyte uncoupling by either *St*-HA′ase or HA_6 on the synthesis and assembly of matrix molecules was analyzed in normal human articular chondrocytes. mRNA copy numbers of HAS-2, CD44, and aggrecan were determined by competitive, quantitative reverse transcriptase–polymerase chain reaction (RT–PCR). HA deposition in cell-associated matrix was determined using biotinylated HA binding protein (HABP). The cell-associated matrix assembly was visualized by particle exclusion assay.

Materials and methods

Tissue acquisition

Human articular cartilage from ten donors, with no known joint disease or diseases that might affect joint degeneration, was obtained within 24 h of death, through the Regional Organ Bank of Illinois according to that institution's protocol and with institutional approval. Noncalcified articular cartilage was dissected from the talus of donors.

Chondrocyte cultures

Full-thickness articular cartilage slices were dissected under aseptic conditions and then subjected to sequential pronase/collagenase (Calbiochem, San Diego, CA, USA, and Boehringer Manheim, Indianapolis, IN, USA, respectively) digestion to liberate chondrocytes from the tissue, as described previously.[15] Isolated chondrocytes were cultured in alginate (Keltone LV, 1.2% in 150 mM NaCl; Kelco, Chicago, IL, USA) beads, as described previously.[16] The beads were maintained in DMEM/Ham's F-12 medium + 5% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO, USA) and 25 μg/ml ascorbate for 2 days to recover. After 2 days in culture, the chondrocytes in the alginate beads were treated with 2 units/ml *St*-HA′ase (Sigma) for 24 h, or 250 μg/ml HA_6 (prepared as described by Knudson) for 3 days. After the treatment with *St*-HA′ase or HA_6, the medium was changed to normal complete DMEM, and the chondrocytes were cultured for a further 48 h (*St*-HA′ase) or 3 days (HA_6).

RNA isolation

Total RNA was isolated from alginate-cultured human chondrocytes that had been treated with or without *St*-HA′ase, or in the presence or absence of HA_6. Chondrocytes were first released from alginate by treatment with sodium citrate,[12] and extracted for total RNA using Trizol (Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions. RNA was dissolved in diethyl pyrocarbonate-treated H_2O, and the concentration of each RNA sample was measured by its absorbance at 260 nm.

Competitive RT–PCR

Total cytoplasmic RNA was subjected to reverse transcription and quantitative competitive PCR. Briefly, 0.25 μg total RNA was converted to cDNA using Molony murine leukemia virus reverse transcriptase (Perkin–Elmer, Norwalk, CT, USA) in the presence of 0.15 μM HAS-2, CD44, or aggrecan-specific downstream primers (HAS-2, 5′-TTT CTT TAT GTG ACT CAT CTG TCT CAC CGG-3′, CD44, 5′-AAC CGC GAG AAT CAA AGC CAA GGC C-3′; aggrecan, 5′-CTC CAC TGC CTG TGA AGT CAC CAC-3′). DNA fragments that share the same primer template sequence with the target cDNA, but contain a completely different, smaller or larger, intervening sequence, were prepared and used as DNA internal standards.[8,17–19] Aliquots of sample cDNA mixed with serial dilutions of DNA mimics were coamplified as templates in the presence

of downstream primers and 0.15 μM upstream primers for HAS-2, CD44, or aggrecan (HAS-2 upstream, 5′-ATT GTT GGC TAC CAG TTT ATC CAA ACG G-3′; CD44 upstream, 5′-GAT CCA CCC CAA TTC CAT CTG TGC-3′; aggrecan upstream, 5′-GCA CCA TGC CTT CTG CTT CCG AG-3′), in a PCR mixture consisting of 2 mM magnesium chloride, 200 μM deoxyribonucleotide, and 2.5 units of AmpliTaq DNA polymerase. The DNA was denatured by heating at 95°C for 2 min, followed by 23 cycles of 1 min each at 95°C, annealing at 60°C, and extension at 72°C for 1 min (Perkin–Elmer thermocycler). This reaction was followed by a final elongation step that lasted 5 min at 72°C. The amplified products were analyzed by electrophoresis on 1.5% agarose gels followed by staining with SYBR green I. The stained products were scanned and quantified using a fluoroimaging system (Molecular Dynamics).

In order to determine that all samples contained equivalent amounts of RNA (or to normalize the results if there were small differences), in a separate set of reactions, total RNA from samples were coamplified in the presence of serial dilutions of an RNA internal standard (mimic) prepared for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH RNA mimic shares the same primer template sequence but contains a smaller intervening sequence. Samples containing 0.25 μg sample total RNA were coreverse transcribed with 2-fold serial dilutions of GAPDH RNA mimic in the presence of 0.15 μM GAPDH-specific downstream primer (5′-TTA CTC CTT GGA GGC CAT GTG GGC C-3′). The sample and mimic cDNA products were then coamplified in the presence of the GAPDH-specific downstream primer together with 0.15 μM upstream primer (5′-ACT GCC ACC CAG AAG ACT GTG GAT GG-3′) using PCR conditions as described for HAS-2 amplification.

Hyaluronan staining with HABP

Chondrocytes released from alginate were fixed with 2% paraformaldehyde buffered with phosphate buffer saline (PBS) at room temperature for 2 h. The cells were then treated with 0.3% H_2O_2 in 30% methanol for 30 min at room temperature to block the internal peroxidase activity, and then incubated with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were then incubated with 2.0 μg/ml of a biotinylated HA binding protein (HABP; Seikagaku America, Ijamsville, MD, USA) probe for 2 h at room temperature, followed by a streptavidin–peroxidase reagent (Vectastain kit) and a diaminobenzidine-containing substrate solution (Sigma Fast Dab). As a control, chondrocytes were pretreated with 5 units/ml *St*-HA′ase for 1 h at 60°C.

Particle exclusion assay

Cell-associated pericellular matrices were visualized using a particle exclusion assay.[4] Briefly, following treatment of alginate cultures of chondrocytes with or without *St*-HA′ase for 24 h, or HA_6 for 3 days, the chondrocytes were released from the alginate beads and transferred to a six-well, flat bottom, tissue culture plate (Falcon), and “splatted” onto the substratum by centrifugation at 500 *g* for 15 min in an Omnifuge RT (Baxter Scientific, McGaw Park, IL, USA) microtiter plate holder.[20] The supernatant medium was removed and replaced with a 0.75-ml suspension of formalin-fixed erythrocytes (10^8 per ml) in PBS containing 0.1% BSA. The particles were allowed to settle for 15 min. The cells were observed and photographed with an inverted phase-contrast microscope with Varel optics (Zeiss, Thornwood, NY, USA).

Results

Effect of *St*-HA′ase or HA_6 on chondrocyte mRNA expression

Previous studies in this laboratory have suggested that cartilage CD44, aggrecan, and HAS activity are regulated predominantly at the level of transcription.[7,8,17] Therefore, changes in mRNA expression of these components generally translate into changes in protein expression or enzyme activity. Thus, to investigate the mechanisms of *St*-HA′ase- and HA_6-induced changes in matrix molecules, the expression of HAS-2, CD44, and aggrecan mRNA was determined by quantitative, competitive RT–PCR analysis. Figure 1 depicts a representative competitive RT–PCR analysis at the 24-h time point. Three independent experiments at this time point were averaged, and the mRNA copy number was expressed after normalization of the mRNA copy number of GAPDH. The effects of HA_6 treatment for 3 days on chondrocyte mRNA expression was determined by the same competitive RT–PCR, and the mRNA copy number was expressed per GAPDH mRNA copy number. As can be seen in Table 1, HAS-2 mRNA was up-regulated (1.8-fold) following treatment with *St*-HA′ase. Aggrecan and CD44 mRNA were not stimulated at the 24-h time point. In HA_6-treated chondrocytes, both aggrecan and HAS-2 mRNA were up-regulated (1.8-fold and 2.0-fold, respectively), compared with control cultures, whereas CD44 mRNA was not affected by the treatment with HA_6. Changing the medium including *St*-HA′ase or

Table 1. Effect of *St*-HA′ase or HA_6 on HAS-2, aggrecan, and CD44 mRNA copy number expressed by human normal articular chondrocytes[a]

	HAS-2	Aggrecan	CD44
St-HA′ase	11.7 ± 1.8	430 ± 74	8.06 ± 0.34
Control	6.8 ± 1.8	403 ± 11	6.84 ± 0.40
HA_6	4.84 ± 1.80	197 ± 21	3.63 ± 0.54
Control	2.32 ± 1.15	112 ± 34	3.56 ± 0.38

[a] Values are mean ± SD copy number of mRNA expressed by the chondrocytes per 10^3 mRNA copies of expressed glyceraldehyde-3-phosphate dehydrogenase as determined in three separate experiments

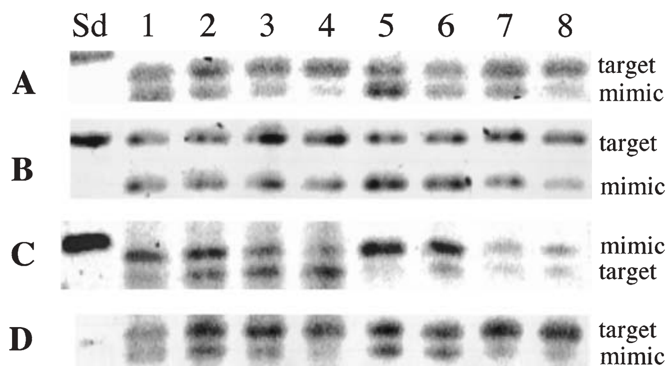

Fig. 1. Representative quantitative competitive reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), CD44, HAS-2, and aggrecan mRNA levels expressed by human articular chondrocytes treated with 2 units of *St*-HA′ase. Aliquots (0.25 μg) of total RNA isolated from 24-h cultures of human articular chondrocytes treated with or without 2 units/ml *St*-HA′ase were reverse-transcribed and PCR-amplified in the presence of internal RNA standards for GAPDH (1, 0.5, 0.25, or 0.125 femtomoles of mimic), DNA standards for CD44 (8, 4, 2, or 1 attomoles for mimic), HAS-2 (30, 15, 7.5, or 3.75 attomoles of mimic), and aggrecan (0.2, 0.1, 0.05, or 0.025 attomoles for mimic). The products were resolved on 1.5% agarose gels and visualized by SYBR green I staining. Lanes 1–4 represent amplification of total RNA derived from 24-h *St*-HA′ase-treated cultures. Lanes 5–8 represent RNA derived from 24-h controls (untreated cultures). The relative band intensities corresponding to the target and mimic PCR products were quantified using ImageQuaNT software (Molecular Dynamics), and the log of the ratio of the amount of target to mimic PCR products was plotted as a function of the log of the amount of mimic added to the reaction. **A** GAPDH target, 465 bp; GAPDH mimic, 370 bp. **B** CD44 target, 587 bp; CD44 mimic, 379 bp. **C** HAS-2 target, 409 bp; HAS-2 mimic, 523 bp. **D** aggrecan target, 620 bp; aggrecan mimic, 512 bp

HA_6 to normal complete DMEM resulted in a return to control levels of mRNA expression by the time point of 24 h (for *St*-HA′ase) or 2 days culture (for HA_6) (data not shown).

HABP staining

Changes in the accumulation of HA can be quantified using binding protein (HABP) that binds HA with high affinity and specificity, as also antibodies.[21] Therefore, a biotinylated HABP probe was used to visualize HA within the cell-associated matrix of human articular chondrocytes released from alginate beads following culture with or without *St*-HA′ase, or in the presence or absence of HA_6. As shown in Fig. 2B, *St*-HA′ase treated chondrocytes released from alginate beads after 24 h culture showed decreased HABP-positive staining, whereas the control cells displayed the intense staining of cell-associated HA (Fig. 2A). The *St*-HA′ase-treated chondrocytes showed that at the 24-h time point after changing the medium to complete DMEM/10%FBS HABP-positive staining appeared to be more intense (Fig. 2C), suggesting the recovery of the chondrocytes to synthesize HA, which was comparable to the up-regulation of HAS-2 mRNA expression at 24 h. HA_6-treated chondrocytes exhibited decreased HABP-positive staining

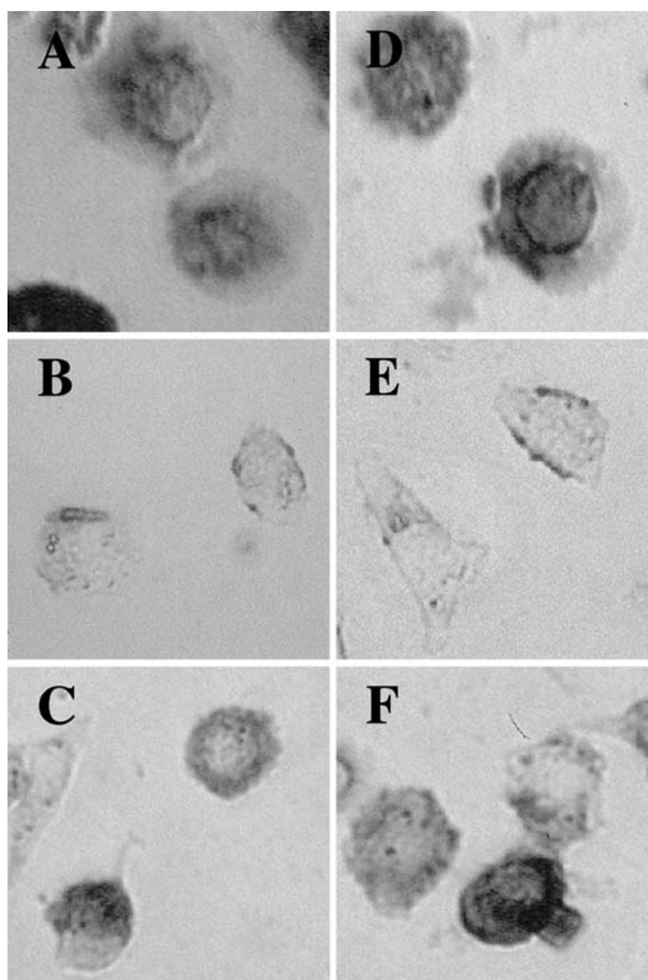

Fig. 2. The effect of *St*-HA′ase or HA_6 on HA deposition by human articular chondrocytes. For the determination of HA, human chondrocytes were first released from the alginate beads, fixed in 2% paraformaldehyde, and stained for HA using a biotinylated HA binding protein (HABP) probe, followed by streptavidin peroxidase and peroxidase substrate. **A** HA accumulation in untreated chondrocytes as a control for *St*-HA′ase-treated chondrocytes. **B** HA accumulation in *St*-HA′ase-treated chondrocytes for 24 h. **C** HA accumulation in recovered chondrocytes after treatment with *St*-HA′ase. **D** HA accumulation in untreated chondrocytes as a control for HA_6-treated chondrocytes. **E** HA accumulation in HA_6-treated chondrocytes for 3 days. **F** HA accumulation in recovered chondrocytes after treatment with HA_6

(Fig. 2E) compared with untreated control chondrocytes (Fig. 2D). HA_6-treated cells also showed the recovery of HABP-positive staining at the time point of 2 days culture in DMEM/10%FBS medium (Fig. 2F), which is in accordance with the stimulation of HAS-2 mRNA expression. Chondrocytes released from alginate beads, again pretreated with *St*-HA′ase, were stained with HABP as negative controls. The cells for negative controls showed almost no HABP positive staining (data not shown).

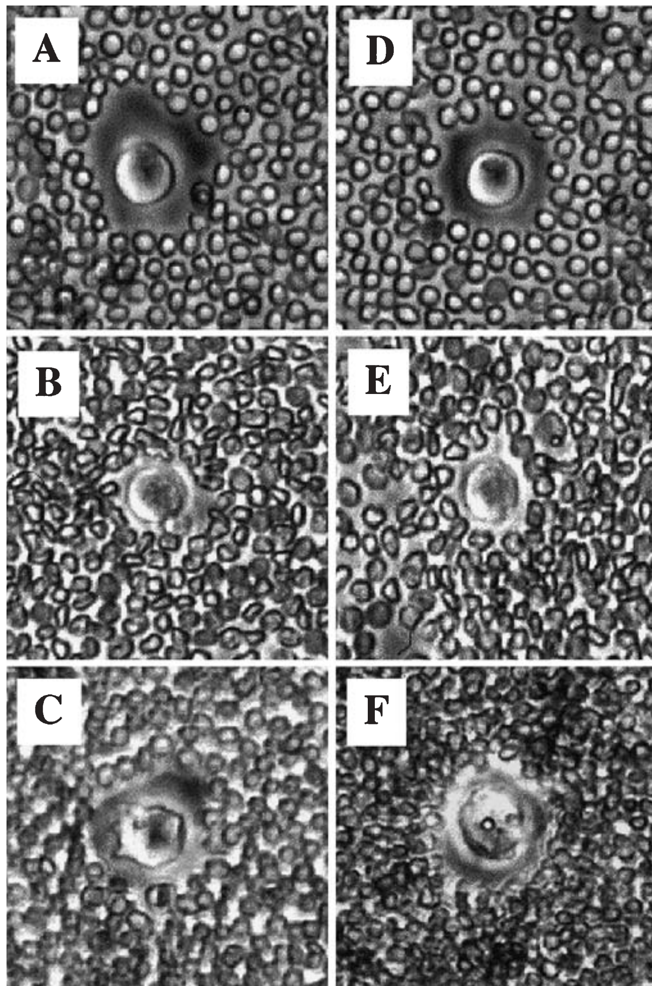

Fig. 3. Effect of *St*-HA′ase or HA_6 on cell-associated matrix assembly by human articular chondrocytes in vitro. Fixed erythrocytes were applied to human articular chondrocytes released from alginate beads cultured for 24 h with (**B**) or without (**A**) 2 units of *St*-HA′ase. After removal of *St*-HA′ase, treated cells displayed recovered cell-associated matrix (**C**). In the absence (**D**) or presence (**E**) of 250 μg/ml HA_6 for 3 days, the chondrocytes were also visualized by particle exclusion assay. The HA_6-treated cells showed recovery of cell-associated matrix after 2 days of culture following the removal of HA_6 (**F**)

Effects of *St*-HA′ase and HA_6 on cell-associated matrix assembly

One of the functions of HA in chondrocytes is to serve as a scaffold for the assembly of a cell-associated matrix. These cell-associated matrices are best visualized surrounding living cells with the use of a particle exclusion assay. As can be seen in Fig. 3A, untreated control chondrocytes exhibited a prominent cell-associated matrix. However, in the *St*-HA′ase-treated cells, these matrices were significantly reduced (Fig. 3B). HA_6 treatment resulted in a substantial decrease in the diameter of cell-associated matrix (Fig. 3E) compared with untreated control chondrocytes (Fig. 3D). The effects of treatment with either *St*-HA′ase or HA_6 on the cell-associated matrix could be reversed after removal of the *St*-HA′ase or HA_6 (Fig. 3C, F, respectively). These data suggest that up-regulation of HAS-2 by these treatments could lead to an increased capacity to assemble cell-associated matrix.

Discussion

Among the many changes that characterize cartilage degeneration, one of the most dramatic is the loss of proteoglycans (PGs), especially aggrecan, from the extracellular matrix. In some experimental systems such as the joint immobilization model, the loss of PG is accompanied by a depletion of HA within the extracellular matrix.[22] Thus, loss of PG may be due to increased PG degradation via elevated matrix metalloproteinase activity,[23] inhibition of PG biosynthesis,[17,23,24] and/or an inhibition of PG retention due to a decrease of HA.[8]

Our previous work has demonstrated that HA plays a critical role for the retention of aggrecan within the cell-associated matrix of chondrocytes.[4,9,25] This matrix can be visualized surrounding living chondrocytes through the use of a particle exclusion assay, or HABP-positive staining of HA. These matrices can be removed completely by treatment with *St*-HA′ase, which suggests a clear dependence on HA for retention of cell-associated matrix. In addition, we have found that these matrices are also removed by incubating the cells with HA oligosaccharides.[5,9,10] These findings indicated that retention of the cell-associated matrix by the chondrocytes requires not only the presence of HA and aggrecan, but also the anchorage of the HA/aggrecan aggregates to the chondrocyte plasma membrane through HA receptors such as CD44.

These findings suggest that successful repair in human cartilage requires not only appropriate increased biosynthesis of the necessary matrix molecules, but also the components necessary to retain and assemble the matrix constituents. A previous study revealed that hyaluronidase up-regulates HA synthase activity in oligodendroglioma cells,[26] but the mechanism of the stimulation is not well known because HASs were not yet identified at that time. The present study has clarified the process of repair by human articular chondrocytes after the treatment with *St*-HA′ase or HA_6, which abrogate the retention and assembly of the extracellular matrix. Particle exclusion assay indicated that 24 h culture in the presence of *St*-HA′ase depleted the cell-associated matrix formation of the chondrocytes. We determined that *St*-HA′ase treatment caused the up-regulation of HAS-2 mRNA expression, which predominantly synthesizes HA in human articular cartilage, at the 24-h time point, whereas there is no change in aggrecan or CD44 mRNA. Given that *St*-HA′ase will digest HA of cell-associated matrix, but not CD44 on cell surfaces of the chondrocytes, there may be no mechanism of positive feedback for CD44 synthesis. Aggrecan mRNA expression was increased at the 48-h time point of the culture (data not shown), suggesting that at 24 h under the effects of *St*-HA′ase, chondrocytes will first prepare the scaffold of the cell-associated matrix by the synthesis of

HA, and then up-regulation of aggrecan biosynthesis will occur as the next step of the repair. HA_6 treatment of the chondrocyte for 3 days culture also abrogates the cell-associated matrix assembly, leading to the stimulation of HAS-2 mRNA expression as well as aggrecan, but not of CD44 mRNA expression. HA, which binds to the chondrocytes through cell surface receptors, is suggested to be displaced by HA_6,[9,10] and thus the extracellular matrix components could not assemble appropriately. However, the effects of HA_6 on the chondrocytes take longer than *St*-HA′ase, which can explain the up-regulation not only of HAS-2 mRNA, but also of aggrecan for the recovery of cell-associated matrix. If HA_6 replaces HA on a cell surface receptor, such as CD44, no positive feedback on CD44 synthesis will occur, and this is in agreement with the finding that CD44 mRNA was not affected by the treatment with HA_6.

One question that arises is whether the reparative responses by the chondrocytes following a loss of the cell-associated matrix interactions was initiated by receptor signaling or by a disruption of the cell architecture, such as intracellular cytoskeletal rearrangements following loss of the HA and PG. The particle exclusion assay and HABP staining demonstrate that cells treated with *Streptomyces* hyaluronidase or HA_6 showed no apparent change in shape. This does not mean that subtle cytoskeletal rearrangement was not occurring, but the cells did not become fibroblastic in morphology. The treatment with *Streptomyces* hyaluronidase or HA hexasaccharides disrupts the HA–PG cell-associated matrix, but may not damage the collagenous cell-associated matrix. These considerations suggest that reparative responses due to the loss of the cell–matrix interaction might be initiated via receptor signaling.

In these experiments, macroscopically normal (Collins grade 0) articular cartilage was excised from relatively older human,[27] and cultured in alginate beads. It has previously been reported that levels of aggrecan mRNA depend on age, and a translational mechanism controls the synthesis of aggrecan core protein.[28] The degree of matrix recovery varied a little between donors, but the chondrocytes from Collins grade 0 cartilage showed enough increase of HAS-2 mRNA to reconstruct the cell-associated matrix. The chondrocytes from cartilage with a Collins grade >1 or osteoarthritic cartilage should be examined to determine whether the extracellular matrix could be recovered or not. Other experiments concerning the capability of the chondrocytes to retain and assemble the matrix are necessary.

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