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Osteogenic Protein-1 inhibits matrix depletion in a hyaluronan hexasaccharide-induced model of osteoarthritis[1]

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Summary

Objective: To examine the capacity of recombinant osteogenic protein-1 (OP-1) to inhibit the cartilage extracellular matrix damage that follows treatment with hyaluronan hexasaccharides (HA6).

Design: The effects of OP-1 were examined on isolated human chondrocytes grown in alginate beads as well as articular cartilage slices treated with hyaluronan hexasaccharides. Changes in the relative expression of messenger RNA for hyaluronan synthase- 2, aggrecan and CD44 were determined by competitive quantitative reverse transcriptase-polymerase chain reaction. Cartilage proteoglycan biosynthesis was examined by a ^{35}S-sulfate incorporation assay. Cell-associated matrix of human chondrocytes was visualized by the use of particle exclusion assay, and alcian blue staining. Cartilage slices were examined for accumulation of proteoglycan by Safranin-O, and hyaluronan by a specific biotinylated probe.

Results: Combined OP-1 and HA6 treatment resulted in enhanced expression of mRNA for aggrecan and HAS-2, compared to the treatment with HA6 only. This increased expression of aggrecan mRNA was paralleled by an increased synthesis of cartilage proteoglycan especially retained in the cell-associated matrix. Co-treatment with OP-1 inhibited the HA6-induced depletion of cell-associated matrices as well as HA6-induced depletion of hyaluronan and proteoglycan within cartilage tissue slices.

Conclusions: These results demonstrate that OP-1 can abrogate the catabolic events associated with a HA6-induced matrix depletion model of osteoarthritis. The mRNA levels of two major cartilage extracellular matrix components, aggrecan and hyaluronan synthase-2 are enhanced above values obtained by either OP-1 or HA6 treatments alone.

Key words: Cartilage, osteogenic protein-1, Hyaluronan synthase, hyaluronan hexasaccharides, human articular chondrocytes.

Introduction

Osteoarthritis is a degenerative joint disease characterized by the loss of extracellular matrix macromolecules within articular cartilage. The most remarkable and consistent alteration is a depletion of the aggregating cartilage proteoglycan, aggrecan[1]. However, since the retention of aggrecan within the cartilage matrix is dependent on the presence of optimal concentrations of hyaluronan (HA) as well as associated functional HA receptors, CD44 in particular, metabolic alterations in any one of these three molecular components may contribute to the loss of this proteoglycan. A significant decrease of HA has been noted in human cartilage derived from osteoarthritis patients as compared with normal human cartilage[2]. Depletion of HA has been observed in early stages of experimental osteoarthritis in dogs[3]. Reduced joint loading due to splint immobilization in dogs also resulted in a significant decrease of HA in articular cartilage[4]. In the latter unloaded canine cartilage model, decreased synthesis appears to be responsible for the loss of matrix HA[5,6]. To test the role of HA on the maintenance of homeostasis in articular cartilage, a means to affect the specific disruption of HA is needed. We have reported that antisense inhibition of hyaluronan synthase-2 (HAS-2), the principal enzyme involved in HA biosynthesis in articular cartilage, caused a depression of cell-associated matrix formation in human articular cartilage[7]. Previous investigations in our groups have also demonstrated that small HA oligosaccharides with a minimum length of 6 monosaccharides (HA6) effectively competed the binding of HA to CD44[8–10], resulting in the loss of proteoglycans (PGs) and an elevation or activation of gelatinolytic activity as well as aggrecanase activity, effectively mimicking osteoarthritic cartilage[11]. Large sized HA oligosaccharides (i.e., HA with ≥10 monosaccharides) are equally effective in displacing CD44-bound HA. However, these oligosaccharides have the additional capacity to displace non-link protein-stabilized aggrecan from HA filaments[12–14]. Therefore, in order to examine the effects of the disruption of HA specifi-

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cally bound to the CD44 receptor, HA6 or HA8 should be used.

Osteogenic protein-1 (OP-1) also known as bone morphogenetic protein-7 (BMP-7) is a member of the transforming growth factor-β superfamily[15]. BMPs were originally characterized by their capacity to induce the formation of cartilage and bone at heterotopic sites[16,17]. Other studies have demonstrated that recombinant BMP can induce endochondral bone formation[15,18] and promote and maintain the chondrogenic phenotype[19–21]. The latter was indicated by their ability to stimulate proteoglycan synthesis in chick limb bud cell cultures, fetal rat chondroblasts, rabbit and bovine articular chondrocytes[19–21]. Among the family of BMPs, OP-1 has been shown to stimulate proteoglycan synthesis in cultured bovine articular chondrocytes and porcine articular cartilage explants[22,23] in particular, the synthesis of type II collagen and aggrecan, without inducing the synthesis of type I or type X collagen, phenotypic markers of chondrocyte differentiation[24]. Recently, we have demonstrated that OP-1 also stimulates HA and CD44 synthesis in addition to aggrecan. The functional outcome of this upregulation was an increase in the cell-associated matrix associated with human or bovine articular chondrocytes[25,26].

Among the many models attempting to mimic osteoarthritic cartilage, the general approach is to affect an enhancement of cartilage catabolism. For example, with the addition of IL-1 there is an induction of MMP activity and an inhibition of proteoglycan synthesis[27]. Treatment of cartilage tissues with trypsin[28] or collagenase[29] disrupts the cartilage matrix and is followed by enhanced matrix biosynthesis. Addition of small HA oligosaccharides (HA6) to cartilage tissues specifically depletes the HA bound to CD44, leading to 'chondrocytic chondrolysis' in spite of an enhancement of the expression of HAS-2 and aggrecan mRNA[11]. In this study, the effect of OP-1 on the matrix depletion induced by HA oligosaccharides in human articular chondrocytes as well as cartilage slices was examined. HAS-2 and aggrecan mRNA expression were stimulated by the HA6 treatment; however, OP-1 enhanced the mRNA expression above these levels in HA6 cultures. A ^{35}S-sulfate incorporation approach demonstrated that newly-synthesized proteoglycan in cell-associated matrix was upregulated by OP-1 in HA6 treated cultures. Alcian blue staining indicated an accumulation of cell-associated proteoglycan in HA6 treated chondrocytes stimulated with OP-1. This up-regulation also gave rise to prominent increases in functional cell-associated matrices. In cartilage slices, Safranin-O staining revealed that the HA6-induced proteoglycan loss could be prevented by the addition of OP-1, together with an enhanced accumulation of HA. The findings suggest that OP-1 effectively prevents the cartilage matrix depletion in an HA6-induced 'chondrocytic chondrolysis' model.

Materials and methods

TISSUE ACQUISITION

Human articular cartilage from donors without known joint disease was obtained within 24 h of death from the 'Gift of Hope' Organ & Tissue Donor Network (formerly the Regional Organ Bank of Illinois), in accordance with institutional protocol and with institutional approval. The average age of the donors was 59 years ranging from 43 to 69 years of age. Noncalcified articular cartilage was dissected from the talus of donors.

CELL CULTURE

Full thickness articular cartilage slices were dissected under aseptic conditions and then subjected to sequential pronase/collagenase (Calbiochem, San Diego, CA and Boehringer Manheim, Indianapolis, IN, respectively) digestion to liberate chondrocytes from the tissue as described previously[30]. Isolated chondrocytes were cultured in alginate (Keltone LV, 1.2% in 150 mM NaCl; Kelco, Chicago, IL) beads as described previously[31]. The beads were maintained in DMEM/Ham's F-12 medium+5% fetal bovine serum (FBS; Summit Biotechnology, Ft. Collins, CO) and 25 μg of ascorbate per ml for 5 days to recover. After 5 days in culture, the chondrocytes in the alginate beads were treated with 250 μg/ml of HA6 in the presence or absence of 100 ng/ml recombinant human OP-1 (rHuOP-1; Stryker Biotech, Hopkinton, MA) which was stored as a stock solution in 50% ethanol and 0.1% trifluoroacetic acid (TFA) at –20°C. HA6 was prepared as described previously[11]. Briefly, human umbilical cord HA (Sigma, St. Louis MO) was used to generate HA oligosaccharides by testicular hyaluronidase cleavage. After precipitation of heat-inactivated hyaluronidase, the oligosaccharides were dried, dissolved in water, and further characterized by Biogel P-6 chromatography (Bio-Rad, Richmond, CA).

TISSUE CULTURE

Full thickness slices of the human articular cartilage (~10×10×1 mm) were cultured directly in 1.0 ml of medium containing 10% FBS. Following 2 days of culture for recovery, the tissue slices were treated in the presence of HA6 with or without OP-1 in the presence of 5% of fetal bovine serum (FBS). The concentration of HA6 and OP-1 is 250 μg/ml and 100 ng/ml, respectively, and in the range shown previously to elicit significant responses in cultured chondrocytes[11,25]. The culture medium containing HA6 with or without OP-1 was changed every 4 days. Following 14 days of incubation the slices were removed and embedded in O.C.T. embedding compound (Tissue-Tek^{R}, Electron Microscopy Sciences, Washington, PA). Cryostat sections (8.0 μm) were prepared and stained for Safranin-O and HA.

RNA ISOLATION

Total RNA was isolated from alginate-cultured human chondrocytes that had been treated with or without 100 ng/ml of OP-1 in the presence of 250 μg/ml of HA6 for 5 days. Following treatment the chondrocytes were released from alginate by treatment with 55 mM sodium citrate in 150 mM NaCl[31], followed by a digestion with 0.25% trypsin/EDTA for 10 min. The cells were then washed once with phosphate buffered saline (PBS) and extracted for total RNA using Trizol (Life Technologies, Grand Island, NY) and following the manufacturer's instructions. RNA was dissolved in diethyl pyrocarbonate-treated H_2O, and the concentration of RNA in each sample was measured by its absorbance at 260 nm.

COMPETITIVE RT-PCR

Total cytoplasmic RNA extracted from chondrocytes with Trizol reagents was subjected to reverse transcription and quantitative competitive PCR. Briefly, 0.25 μg of total RNA was converted to cDNA using Molony murine leukemia virus reverse transcriptase 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 (i.e., mimics)[25,32,33]. Aliquots of sample cDNA mixed together with serial dilutions of DNA mimics were co-amplified 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 TAC-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 of each 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 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*). The log of the ratio of fluorescence intensity of target to mimic PCR products was graphed as a function of the log of the attomolar concentration of mimic added to the reaction. When the amplified products are equimolar (including minor corrections for differences in size), the initial concentration of the target mRNA is equal to the concentration of mimic molecules.

To determine that all samples contained equivalent amounts of RNA (or to normalize results due to small differences), in a separate set of reactions total RNA from samples were co-amplified 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 of sample total RNA were co-reverse 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 co-amplified 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.

^{35}S-SULFATE INCORPORATION

After 5 days of 250 µg/ml of HA6 treatment with or without 10, 50 or 100 ng/ml of OP-1, chondrocytes in alginate beads were radiolabeled with ^{35}S-sulfate (20 µCi/ml; Amersham Corp, Arlington Heights, IL) for 4 h. After 24 h of chase, the culture media were removed as the 'medium fraction.' The chondrocytes were then released from alginate by treatment with 55 mM sodium citrate in 150 mM sodium chloride[31], centrifuged and divided into two parts: the pelleted fraction representing the cell-associated matrix and supernatant fraction, the further removed matrix[27]. To extract PGs from cell-associated matrix, each fraction was treated for 24 h at 4°C with 4 M guanidine HCl containing the following protease inhibitors at these final concentrations: 10 mM EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, 10 mM N-ethylmaleimide, and 0.5 mM phenylmethylsulfonylfluoride[34]. ^{35}S-labeled PGs in each medium and dissociative extract were quantified by liquid scintillation counting following rapid filtration, as described previously[35]. The ^{35}S-PGs cpm values were normalized to cellular DNA content[35].

ALCIAN BLUE STAINING

Following treatment of alginate cultures of chondrocytes in the presence of 250 µg/ml of HA6 with or without 100 ng/ml of OP-1 for 5 days, the beads were washed with 0.3 M MgCl2 (containing 25 mM NaAcetate, pH 5.6) and then transferred into a staining solution (made with 20 ml washing buffer plus 1 ml Alcian blue solution; kind gift from Dr John Scott, University of Manchester, UK), for 2 h at room temperature[27]. Following two washes the alginate beads were placed directly onto a slide, and compressed down with a coverslip. The whole mount preparations were photographed under bright-field microscopy.

HYALURONAN STAINING WITH HABP

Eight µm cryostat sections of cartilage slices mounted onto glass slides, were fixed with 2% paraformaldehyde buffered with PBS at room temperature for 2 h. Tissue sections were pretreated with 2 units of chondroitinase ABC (at pH 8.0) for 2 h at 37°C for unmasking. The tissue sections were next 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 in PBS for 1 h at room temperature. Sections were next incubated with 2.0 µg/ml of a biotinylated HA binding protein (HABP) probe (Seikagaku America, Rockville, MD) for 2 h at room temperature followed by a streptavidin-peroxidase reagent (*Vectastain* kit, Burlingame, CA) and diaminobenzidine-containing substrate solution (*SIGMA FAST™ DAB*). As a control, tissue sections were pretreated with 5 units/ml *Streptomyces* hyaluronidase for 1 h at 60°C.

PARTICLE EXCLUSION ASSAY

Cell-associated pericellular matrices were visualized using a particle exclusion assay[9]. Briefly, following 250 µg/ml of HA6 treatment of alginate cultures of chondrocytes with or without 100 ng/ml of OP-1 for 7 days, the chondrocytes were released from alginate beads and transferred to six-well, flat bottom, tissue culture plate (*Falcon*), and 'splatted' onto the substratum by centrifugation at 500 ***g*** for 15 min in an Omifuge RT (*Baxter Scientific*, McGaw Park, IL) microtiter plate holder[36]. 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% bovine serum albumin. 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).

STATISTICAL ANALYSIS

Results of gene expression and proteoglycan synthesis studies were expressed as the mean±SD of triplicate

samples. Analysis was performed using student's unpaired 2-tailed *t*-tests. *P* values less than or equal to 0.05 were considered significant.

Results

THE EFFECT OF OP-1 ON CHONDROCYTE GENE EXPRESSION OF AGGRECAN, HAS-2 AND CD44 IN HA6 INDUCED MATRIX DEPLETION MODEL

Previous studies in our laboratory have shown that the assembly of chondrocyte cell-associated matrices depend on the presence of three components namely, aggrecan, HA and CD44[7,8,37]. To investigate the effects of OP-1 in a HA6 induced matrix depletion model on the expression of these molecules, the mRNA levels of aggrecan, HAS-2 and CD44 were determined by quantitative competitive RT-PCR analysis. Fig. 1A show a representative competitive RT-PCR analysis for CD44, HAS-2 and aggrecan respectively, after 5 days of culture. Absolute copy numbers of GAPDH, CD44, HAS-2 and aggrecan in a representative control culture were, 275 attomoles, 1.05 attomoles, 1.81 attomoles and 112 attomoles/0.25 μg of total RNA, respectively. Data from three independent experiments were averaged and the change in mRNA copy number due to OP-1 plus HA6 or HA6 alone versus mRNA levels in untreated control chondrocytes was expressed as a ratio. As shown in Fig. 1B, HAS-2 and aggrecan mRNA copy number was up-regulated by HA6 treatment compared with untreated control cultures (~2-fold increase). The CD44 mRNA level was slightly up-regulated (1.3-fold increase, P=0.48). Addition of OP-1 to the cultures in the presence of HA6 significantly stimulated the expression of HAS-2 and aggrecan mRNA levels compared with HA6 alone (2.2-fold increase, P=0.046 and 2.5-fold increase, P=0.036, respectively). The CD44 mRNA level was not significantly affected by the addition of OP-1 to HA6 cultures. These mRNA levels were all normalized by GAPDH competitive RT-PCR analysis.

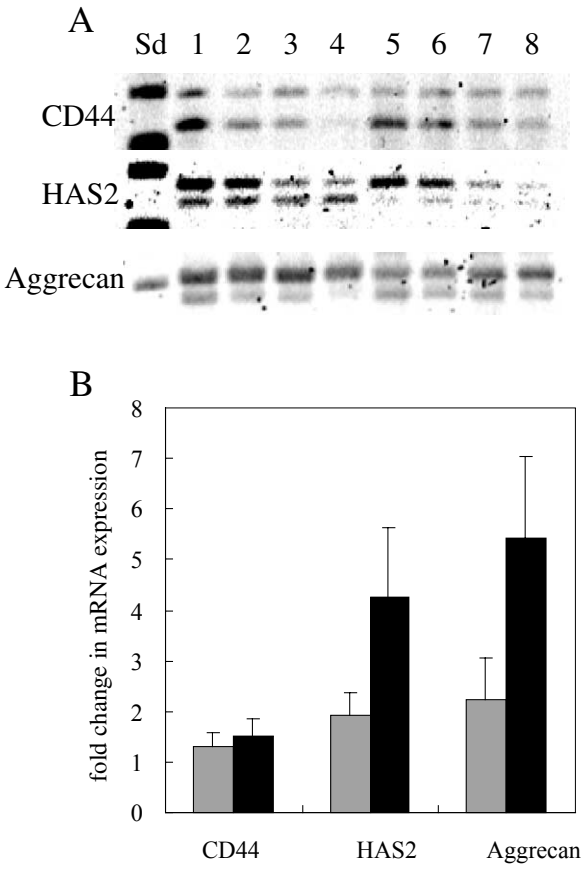


Fig. 1. The effect of OP-1 on CD44, HAS-2, aggrecan mRNA expression by human articular chondrocytes treated with HA6. Aliquots of total RNA derived from human articular chondrocytes cultures treated by 250 μg/ml of HA6 with or without 100 ng/ml of OP-1 for 5 days, were reverse transcribed and PCR amplified for 23 cycles in the presence of internal DNA standards for CD44 (4.0, 2.0, 1.0 and 0.5 attomoles of mimic), HAS-2 (30.0, 15.0, 7.5 and 3.75 attomoles of mimic) or, aggrecan (0.2, 0.1, 0.05 and 0.025 femtomoles of mimic). The products were separated on 1.5% agarose gel and visualized by SYBR green I staining. Panel A, lanes 1-4 show RNA derived from representative 5-day HA6 plus OP-1 treated cultures; lanes 5-8 show RNA derived from 5-day HA6 treated cultures. Lanes Sd represent 603-bp and 310-bp φX174/*Hae* III DNA marker bands. CD44 target 587 bp and mimic 379 bp; HAS-2 target 409 bp and mimic 523 bp; aggrecan target 620 bp and mimic 512 bp. In panel B, after normalization with competitive RT-PCR using GAPDH RNA mimic, the ratio of mRNA copy numbers, of cultures derived from OP-1 plus HA6 and control cultures (black bars), or HA6 and control cultures (light gray bars) were plotted for each molecule. Data represent the mean±standard deviation of triplicate experiments.

THE EFFECT OF OP-1 ON PROTEOGLYCAN BIOSYNTHESIS IN HA6 INDUCED MATRIX DEPLETION MODEL

While HA oligosaccharides have been shown to induce chondrocytic chondrolysis, there is also concomitant induction of proteoglycan and HA biosynthesis[11]. However, in the presence of the HA6, the newly synthesized proteoglycan is not retained in the cell-associated matrix, because the presence of the HA6 blocks the HA-binding activity of CD44, resulting in a decrease of functional cell-associated matrix[38]. To explore the effects of OP-1 on proteoglycan synthesis in the HA6 matrix depletion model, HA6-treated chondrocytes in the presence or absence of OP-1 were pulse-labeled with ^{35}S-sulfate in alginate bead cultures. There was a significant increase in the total amount of PGs synthesized with treatment of HA6 and OP-1 compared to with HA6 alone (P=0.0048). In the cell-associated matrix, the amount of newly synthesized PGs of cells treated with OP-1 and HA6 treatment was significantly higher than that in HA6 treatment alone (P=0.003). Interestingly, newly synthesized PG in the cell-associated matrix depleted by HA6 returned to control level with the addition of OP-1 (Fig. 2A). The effect of OP-1 on proteoglycan synthesis is dose-dependent and the distribution of the newly synthesized PG is prominent in the cell-associated matrix (Fig. 2B). In the further-removed matrix and medium there was not a significant difference in newly synthesized PGs between control and treated chondrocytes. However, in the cell-associated matrix fraction, treatment of chondrocytes with 50 and 100 ng/ml of OP-1 together with HA6 significantly stimulated the synthesis of PGs as compared to HA6 alone P=0.0016 and P=0.0006, respectively). No significant differences were observed in chondrocytes treated with HA6 and 10 ng of OP-1 (P=0.87).

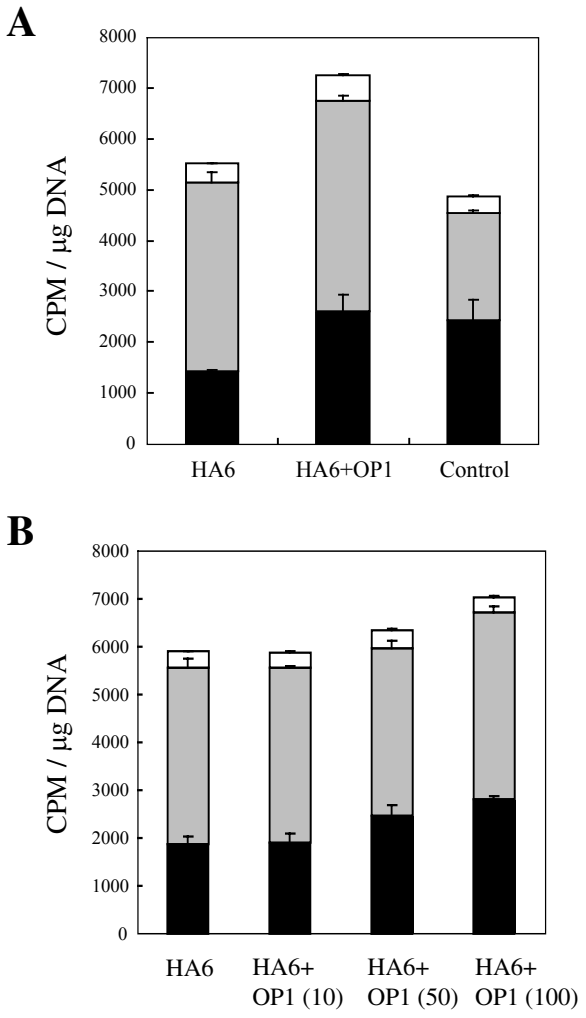


Fig. 2. The effect of OP-1 on PG synthesis and cellular distribution in cultures of human articular chondrocytes treated with HA6. Panel A, five days after the initial addition of HA6 or HA6 plus OP-1, the cultures were pulse-labeled with ^{35}S-sulfate for 4 h and chased for an additional 24 h. The medium (white bars), further-removed matrix (light gray bars) and cell-associated matrix (black bars) pools were collected, and the specific incorporation of ^{35}S-sulfate into PGs was determined using a rapid filtration method. The level of ^{35}S-sulfate labeled PGs synthesized was normalized to cellular DNA content. Values represent the average±SD of triplicate experiments; P=0.0048 for difference between OP-1 plus HA6 and HA6 treatment bars in total amount, P=0.003 between OP-1 plus HA6 and HA6 only treatment bars in cell-associated matrix. Panel B depicts the dose-dependent effects of OP-1 (10, 50 or 100 ng/ml) on ^{35}S-sulfate incorporation for HA6 treated chondrocyte cultures.

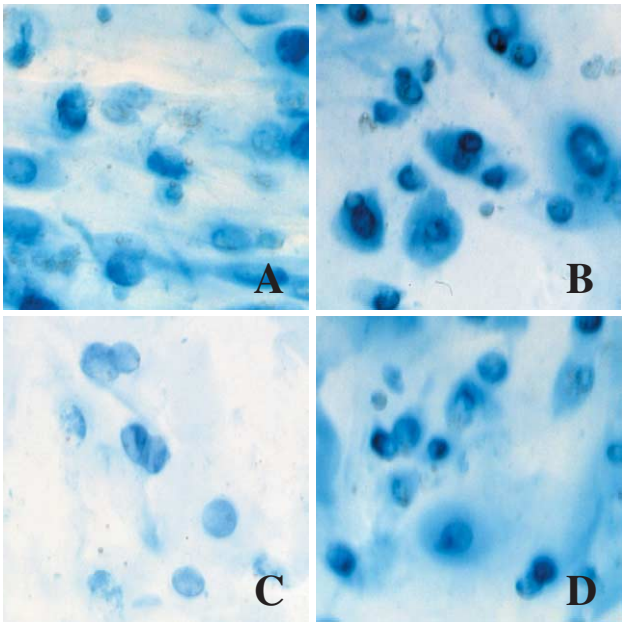


Fig. 3. Alcian blue staining of alginate bead chondrocyte cultures. Panel A, control untreated human articular chondrocytes cultured in alginate beads show a moderate alcian-blue positive matrix. Panel B, 100 ng/ml of OP-1 treated human articular chondrocytes show a prominent increased alcian-blue positive matrix. Panel C, human articular chondrocytes cultured in alginate beads in the presence of 250 µg/ml HA6 show an overall decrease in alcian-blue positive matrix. Panel D, HA6 treated human articular chondrocytes in the presence of OP-1 show a recovered alcian-blue positive matrix compared to the chondrocytes treated with HA6 only.

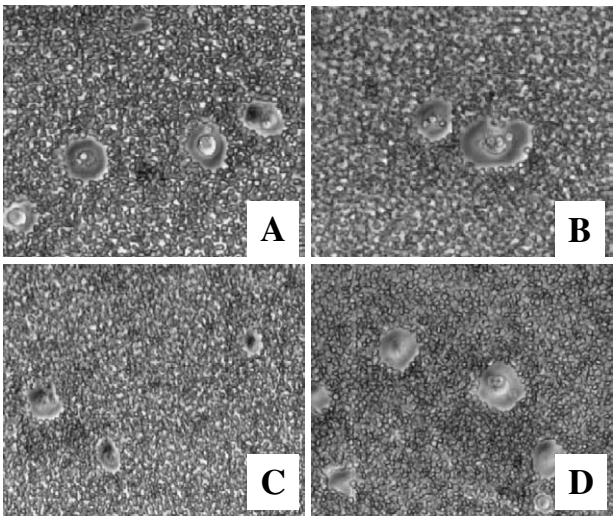
THE EFFECT OF OP-1 ON CHONDROCYTE CULTURES IN HA6 INDUCED MATRIX DEPLETION MODEL

Isolated human articular chondrocytes cultured in alginate beads assembled an alcian blue-positive matrix (Fig. 3A). The staining for proteoglycan was enhanced by treatment with OP-1 (Fig. 3B). After incubation for 7 days in the presence of HA_6, the alginate beads cultures showed a substantial decrease in alcian blue staining for PGs (Fig. 3C). However, when the chondrocyte cultures were co-treated with OP-1 and HA6, the alcian blue positive staining was again prominent, even somewhat enhanced above the control level (Fig. 3D). This intense alcian blue staining in the cell-associated region is in accordance with the stimulated proteoglycan synthesis in the cell-associated matrix pool (Fig. 2).

In the presence of calcium chelators the alginate gel depolymerizes and the chondrocytes can be collected as single cells. In this manner, following centrifugation of the single-cell suspension to attach the chondrocytes to a culture plastic substratum, the retained cell-associated matrix can be visualized on living cells using a particle exclusion assay. As shown in Fig. 4A, control chondrocytes grown for 7 days in the presence of FBS all exhibited a prominent cell-associated matrix – a matrix whose size can be substantially enhanced by treatment with OP-1 (Fig. 4B, 4E) as previously reported[25]. In HA6 treated chondrocytes, the cell-associated matrix size was nearly absent from around the cells (Fig. 4C) resulting in a matrix area-to-cell area equal to 0.2 following morphometric analysis (Fig. 4E). However, co-treatment of chondrocytes with OP-1 in the presence of HA6 prevented the reduction in cell-associated matrix size observed in HA6 matrix depleted model (Fig. 4D). The morphometric ratio under these conditions was similar to that of control chondocytes (Fig. 4E).

THE EFFECT OF OP-1 ON CARTILAGE MATRIX RECOVERY IN HA6 INDUCED MATRIX DEPLETION MODEL

Slices of human articular cartilage isolated directly from dissection were cultured in the presence of HA6 with or without OP-1. Treatment of human articular cartilage with



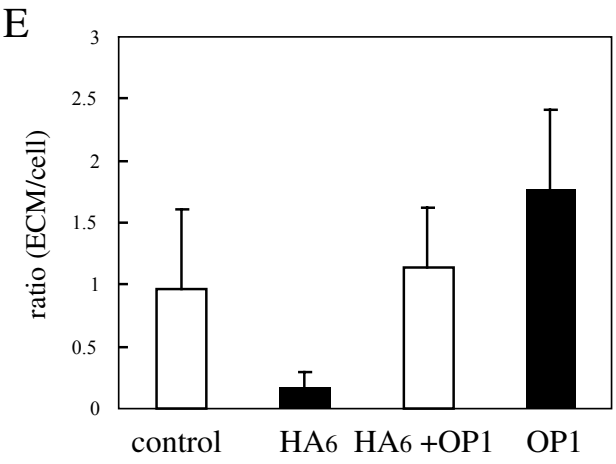


Fig. 4. The effect of OP-1 on the cell-associated matrix of human articular chondrocytes treated with HA6. Human articular chondrocytes were cultured in alginate beads in the presence of 5% FBS for 7 days, released from alginate and transferred into 6-well culture plates. Fixed erythrocytes were applied to the plated chondrocytes to visualize the cell-associated matrix. Panel A, control untreated chondrocytes; Panel B, chondrocytes treated with 100 ng/ml of OP-1; Panel C, chondrocytes treated with 250 μg/ml of HA6 and, Panel D, chondrocytes treated with OP-1 in the presence of HA6. Panel E depicts a morphometric analysis of (matrix area-cell area)/cell area for each of the conditions shown in panels A-D. Bars represent the mean±standard deviation of 10 cells per condition. Calculation of *P*-values demonstrated significant differences (2-tailed student's *t*-test) between control and HA6=0.026 and; between HA6 and (HA6+OP-1)=0.002. Differences between OP-1 and (HA6+OP-1)=0.124 and; between control and (HA6+OP-1)=0.638 were not significant.

250 μg/ml of HA6 for 14 days resulted in the prominent loss of Safranin O positive matrix (Fig. 5A). However, 100 ng/ml of OP-1 protected the cartilage from loss of PG due to HA6 induced matrix-depletion (Fig. 5B). HA plays an important role to maintain the cell-associated matrix in articular cartilage, giving a scaffold to aggrecan, making proteoglycan aggregates and binding chondrocytes via cell surface receptor, CD44. HA accumulation in the cartilage matrix was further analyzed using a specific biotinylated HABP probe for HA. Staining for HA in normal untreated cartilage is shown in the inset of Fig. 5C – staining that was almost totally lost upon treatment with HA6 (Fig. 5C and 5E). Addition of OP-1 in the HA6 induced matrix depletion model stimulated HA accumulation, both in the superficial/upper layer (Fig. 5D) and in deeper layers of articular cartilage (Fig. 5F).

Discussion

Among the many changes that characterize cartilage degeneration, one of the most dramatic events is the loss of proteoglycans, especially aggrecan, from the extracellular matrix. Loss of PG may be due to increased PG degradation via elevated matrix metalloproteinase activity[39], inhibition of PG synthesis[35,39,40], and inhibition of PG retention due to changes in HA metabolism[7]. Our previous work demonstrated that treatment of chondrocytes in cell culture with HA6 results in displacement of the proteoglycan-rich cell-associated matrix, presumably by uncoupling the interactions of HA with its principal receptor, CD44[8,9]. Treatment of cartilage explants with HA6 caused a dramatic loss of proteoglycan in cartilage extracellular matrix and induced chondrocytic chondrolysis, including activation of gelatinolytic and aggrecanase activity. HA6 also induced an increase of proteoglycan synthesis, including elevation of aggrecan and HAS-2 mRNA. However, this increase does not result in the increase of functional cell-associated matrix[11]. This finding suggests that successful repair in human cartilage requires not only appropriate increased synthesis of matrix molecules but also the components necessary to retain and assemble the matrix constituents.

In the current study, OP-1 facilitated the recovery of a cell-associated matrix in cultures of human chondrocytes or cartilage explants that was disrupted by treatment with HA6. In the short term, continued exposure of the cells to high concentrations of HA6 would prevent retention of newly synthesized HA or aggrecan. However, after 7 days of incubation there is little HA6 remaining to effect direct displacement from the CD44. The lack of matrix retention in the HA_6-treated cultures is likely due to the continuation of induced catabolic cascade events. For example, in adult bovine cartilage slices treated with HA6, chondrocytic chondrolysis events continue even when the HA oligosaccharides are washed out of the tissues. Thus, it is likely that the presence of OP-1 together with HA6 acts to prevent or inhibit the signaling events that initiate the degradative process. In previous studies we demonstrated that treatment of chondrocytes or cartilage slices with IL-1α also resulted in the activation of a catabolic-like cascade including the loss of cell-associated matrices surrounding individual cells. All of these events occurred even though the levels of CD44 and HAS-2 were elevated[27,41]. We speculated that the increased CD44 was functional but now being used for HA endocytosis rather than matrix retention. The opposite results were obtained when CD44 expression was upregulated by the treatment of chondrocytes with OP-1[25,26]. In this case the increased CD44 appeared to contribute to enhanced cell-associated matrix retention. Thus in this study, when OP-1 and HA6 are added together, the intracellular mechanisms that regulate CD44 function may now be shifted toward matrix retention as opposed to HA internalization, resulting in enhanced HA and aggrecan retention. This may explain why addition of OP-1 to chondrocytes co-treated with HA6 resulted in enhanced

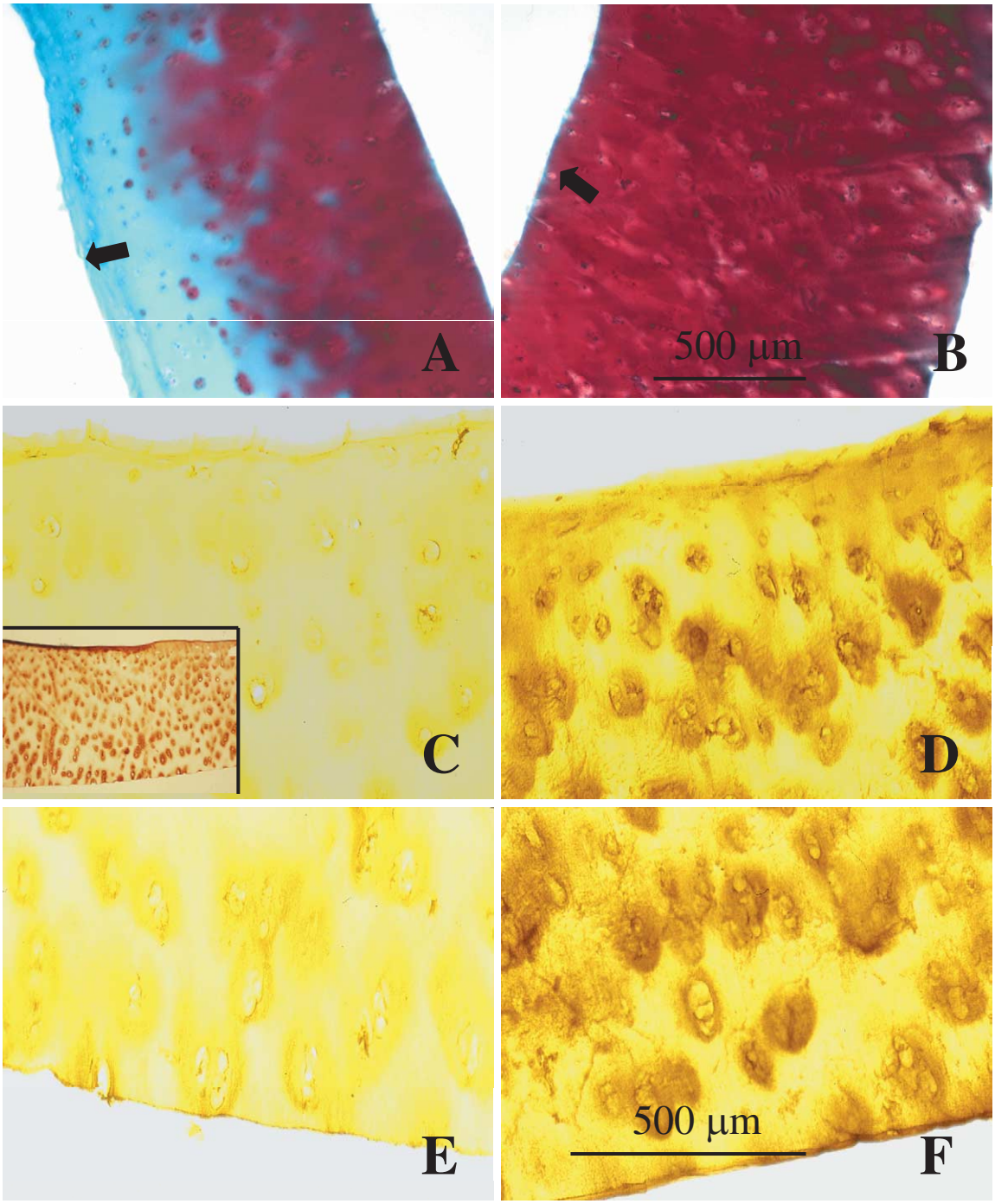


Fig. 5. The effect of OP-1 on PG or HA accumulation by human articular cartilage slices treated with HA6. Human articular cartilage slices were treated without (A, C, E) or with (B, D, F) 100 ng/ml of OP-1 in the presence of 250 μg/ml of HA6 for 14 days. Cartilage slices were stained with Safranin-O/fast green for PGs (A, B), or with biotinylated HABP probe for HA (C-F). Panels C, D depict the superficial/upper layers of cartilage and; panels E, F the deeper layers of cartilage. Untreated control cartilage stained with the HABP probe is shown in the inset of C. Arrows indicate superficial layer of the cartilage in panels A and B. The superficial surface is to the top in panels C-F.

^{35}S-sulfate labeled proteoglycan accumulation and retention in the cell-associated matrix in addition to enhanced overall biosynthesis.

Other studies have demonstrated that OP-1 can also abrogate IL-1β-induced suppression of ^{35}S-sulfate labeled proteoglycan synthesis[42]. This study examined the potential of OP-1 to module proteoglycan synthesis, but did not examine other extracellular molecules, such as HA. In the current study, not only were aggrecan, HAS-2 and CD44 mRNA upregulated, there was also a metabolic change that made the chondrocytes 'permissive' and capable of utilizing these components to assemble the functional HA-rich cell-associated matrix. This permissive environment may be due to inhibition of endogenous proteolytic activity,

inhibition of oxygen radical release or, changes in cytoskeletal-CD44 interactions that inhibit HA endocytosis yet retain extracellular HA binding. In summary, in the HA6 model, wherein there is cartilage matrix depletion but no inhibition of aggrecan biosynthesis (unlike the IL-1 model) functional matrix retention still does not occur unless agents such as OP-1 are present. Thus, attempts to facilitate enhanced matrix biosynthesis may be of less importance than altering the 'permissive' retention capacity of chondrocytes such as occurs in the presence of OP-1. This may explain why phases of attempted repair as seen during the early stages of osteoarthritis inevitably fail.

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