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A stable isotope method for the simultaneous measurement of matrix synthesis and cell proliferation in articular cartilage *in vivo*

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Summary

Objective: Measurements of cell proliferation and matrix synthesis in cartilage explants have identified regulatory factors [e.g., interleukin-1 (IL-1)] that contribute to osteoarthritis and anabolic mediators [e.g., bone morphogenic protein-7 (BMP-7)] that may have therapeutic potential. The objective of this study was to develop a robust method for measuring cell proliferation and glycosaminoglycan synthesis in articular cartilage that could be applied *in vivo*.

Methods: A stable isotope-mass spectrometry approach was validated by measuring the metabolic effects of IL-1 and BMP-7 in cultures of mature and immature bovine cartilage explants. The method was also applied *in vivo* to quantify physiologic turnover rates of matrix and cells in the articular cartilage of normal rats. Heavy water was administered to explants in the culture medium and to rats *via* drinking water, and cartilage was analyzed for labeling of chondroitin sulfate (CS), hyaluronic acid (HA) and DNA.

Results: As expected, IL-1 inhibited the synthesis of DNA and CS in cartilage explants. However, IL-1 inhibited HA synthesis only in immature cartilage. Furthermore, BMP-7 was generally stimulatory, but immature cartilage was significantly more responsive than mature cartilage, particularly in terms of HA and DNA synthesis. *In vivo*, labeling of CS and DNA in normal rats for up to a year indicated half-lives of 22 and 862 days, respectively, in the joint.

Conclusions: We describe a method by which deuterium from heavy water is traced into multiple metabolites from a single cartilage specimen to profile its metabolic activity. This method was demonstrated in tissue culture and rodents but may have significant clinical applications.

Key words: Cartilage metabolism, Aggrecan biosynthesis, Hyaluronan biosynthesis, Chondrocyte proliferation, Bone morphogenic protein, Interleukin, Animal studies, Rat.

Introduction

Chief among the degenerative changes associated with osteoarthritis (OA) is the degradation of articular cartilage, the weight-bearing material found at the end of long bones that facilitates the motion of joints. On a molecular level, articular cartilage is a structurally organized connective tissue whose extracellular matrix consists of a cross-linked network of collagen fibers expanded by water and proteoglycans[1]. The primary proteoglycan in articular cartilage is aggrecan, a macromolecule composed of a core protein covalently attached to up to 150 glycosaminoglycan (GAG) chains, primarily chondroitin sulfate (CS) and keratan sulfate[2]. Up to 200 aggrecan molecules associate on a backbone of hyaluronic acid (HA) to form aggregates reaching molecular weights of over 200 MDa[3].

A homeostatic balance between matrix synthesis and degradation is maintained in healthy cartilage by a sparse population of chondrocytes[4], but these metabolic processes are disrupted in degenerative joint diseases, in which a net loss of extracellular matrix components is observed[5]. Measurements of critical metabolic pathways, including proliferation of chondrocytes and synthesis of extracellular matrix components, have identified regulatory factors that contribute to degenerative joint disease, such as the pro-inflammatory cytokine interleukin-1 (IL-1[6]), as well as anabolic mediators that have therapeutic utility, such as the growth factor bone morphogenic protein-7 (BMP-7)[7].

Kinetic measurements of these metabolic pathways have traditionally employed radioactively-tagged precursors. Methods based on radioactive tracers are sensitive and relatively cost-effective, but their use *in vivo* is limited by safety issues, particularly in humans. In addition to safety advantages over radiotracers, stable isotope labeling with mass spectrometric analysis generates unique information based on patterns of isotopic isomers (e.g., mass isotopomer distribution analysis[8,9]) that can provide critical information about biosynthetic processes. Stable isotope labeling with heavy water (^2H_2O) has been used to label a wide range of metabolites *in vivo*[10–14], including DNA[15], lipids[14] and protein[16]. Hydrogen-labeled water is an attractive tracer because it equilibrates quickly and uniformly with body water and the body water pool turns over relatively slowly (~8% a week[17,18]), allowing ^2H_2O to be maintained easily at relatively constant label concentrations[15,18]. Moreover, protons from water enter C–H bonds at specific enzyme-mediated steps in nearly every metabolic pathway, making

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^2H_2O a near universal tracer. We describe here the first implementation of stable isotopes and mass spectrometry, specifically heavy water, for quantifying the synthetic rates of multiple products, including GAGs and cellular DNA, within articular cartilage *in vivo*.

Relatively few studies have attempted to perform direct metabolic measurements on articular cartilage *in vivo*. Measurements in living animals are important because articular cartilage interacts with other tissues that comprise the synovial joint and are important in the pathology of OA, but such interactions are difficult to reproduce *in vitro*[19]. Here, we present a stable isotope-mass spectrometric technique through which a single tracer can be used to quantify multiple aspects of extracellular matrix synthesis and cell proliferation reliably and accurately in a sample of articular cartilage. To validate the method in a well-characterized model, we quantified CS, HA, and DNA synthesis rates in bovine cartilage explants maintained in tissue culture and examined the effects of IL-1 and BMP-7. The methods were then extended to articular cartilage *in vivo* to measure physiologic rates of extracellular matrix synthesis and articular chondrocytes proliferation in normal, growing rats.

Materials and methods

TISSUE CULTURE OF BOVINE CARTILAGE EXPLANTS

Bovine stifle joints with intact joint capsules were obtained within 24 h after slaughter from three 1–3-week old calves (San Jose Valley Veal, San Jose, CA, USA) and three 12–18-month old steers (Animal Technologies, Dallas, TX, USA). Osteochondral blocks were harvested under sterile conditions from the distal femur using a reciprocating saw (Johnson and Johnson, New Brunswick, NJ, USA). Tissue samples were secured to a sledge microtome (Microm GmbH, Walldorf, Germany), and plane-parallel 1 mm thick sections of articular cartilage were obtained after discarding at least 200 μm from the articular surface. For calf tissue, two sequential 1 mm thick cartilage sections were obtained, whereas the adult articular cartilage layer was only thick enough to yield one section of 1 mm thickness. From each of the cartilage sections, 3 mm diameter disks were obtained using a stainless-steel dermal punch (Miltex GmbH, Tuttlingen, Germany). Cartilage disks were maintained at 37°C and 5% CO_2 in a basal medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 μg/ml ascorbate, antibiotics, and either 4 or 8% ^2H_2O (Spectra Stable Isotopes, Columbia, MD, USA). In some samples, recombinant human IL-1α (R&D Systems, Minneapolis, MN, USA) was added to the medium at a final concentration of either 0.5 or 5 ng/ml. IL-1α was chosen because bovine chondrocytes are more responsive to the α- than the β-isoform[20,21]. This concentration range induces a significant loss of tissue sulfated GAG (sGAG) and inhibits sGAG biosynthesis in adult bovine cartilage explants[21,22]. Some cultures included 100 ng/ml recombinant human BMP-7 (R&D Systems, Minneapolis, MN, USA) throughout the culture period, previously shown to stimulate the biosynthesis of sGAG in adult bovine cartilage in the presence of serum[23–25]. Individual cartilage disks were incubated in 1 ml of medium and transferred to new culture wells containing fresh medium every 2 days to minimize the impact of cell migration onto tissue culture plastic. Explant disks were maintained in culture for 5 or 10 days. Spent medium was frozen and analyzed for released extracellular matrix molecules.

TIME COURSE OF LABEL INCORPORATION IN ARTICULAR CARTILAGE OF NORMAL RATS

Fifty male Sprague Dawley rats (initial weights 254–279 g, Charles River, Wilmington, MA, USA) were administered ^2H_2O under isoflurane anesthesia. Intraperitoneal injection of a priming bolus (35 μl/g body weight) consisted of 100% ^2H_2O in sterile 0.9% saline. Drinking water was replaced with water containing 8% ^2H_2O, and animals drank *ad libitum*. Plasma was collected at sacrifice *via* cardiac puncture. The hind legs were removed, stored at −20°C until processing and articular cartilage was then carefully scraped from the surface of the medial tibia plateaus (MTP) using a scalpel, taking care not to penetrate the subchondral bone. All animal experiments received IACUC approval.

ISOLATION OF DNA, CS, AND HA (FIG. 1)

Cartilage was solubilized by overnight digestion with 500 μg/ml proteinase K at 55°C. The digest was then boiled for 10 min to deactivate proteinase K[26] and portions were transferred to microfiltration tubes with 5 kDa cellulose filters (Millipore, Billerica, MA, USA) and centrifuged (60 min, 5000*g*) to retain macromolecular material. A 50 mM acetate (pH 6) including 10 TRU/ml streptomyces hyaluronidase (Seikagaku, Tokyo, Japan) and 0.02% bovine serum albumin (BSA; Sigma–Aldrich, St. Louis, MO, USA) were added, allowed to incubate at 55°C for 2 h, centrifuged (60 min, 5000*g*) and the filtrate was collected. Residual macromolecular material was digested overnight with 0.1 U chondroitinase ABC (Sigma–Aldrich, St. Louis, MO) in 200 ml 50 mM Tris–acetate (pH 8) including 0.01% BSA, and centrifuged. The two solutions containing hyaluronidase or chondroitinase digestion products were hydrolyzed separately by incubation with equal parts 3 N methanolic HCl (1 h, 20°C). DNA was collected by reconstituting the filter retentate with water, then digested to free deoxyribonucleosides as described previously[10].

MEASUREMENT OF MASS ISOTOPOMER ABUNDANCES BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

CS-, HA- and DNA-hydrolysis products were derivatized to the pentofluorobenzyl derivatives and acetylated, as described elsewhere[10]. *N*-Acetyl glucosamine (glcNAc), *N*-acetyl galactosamine (galNAc) and

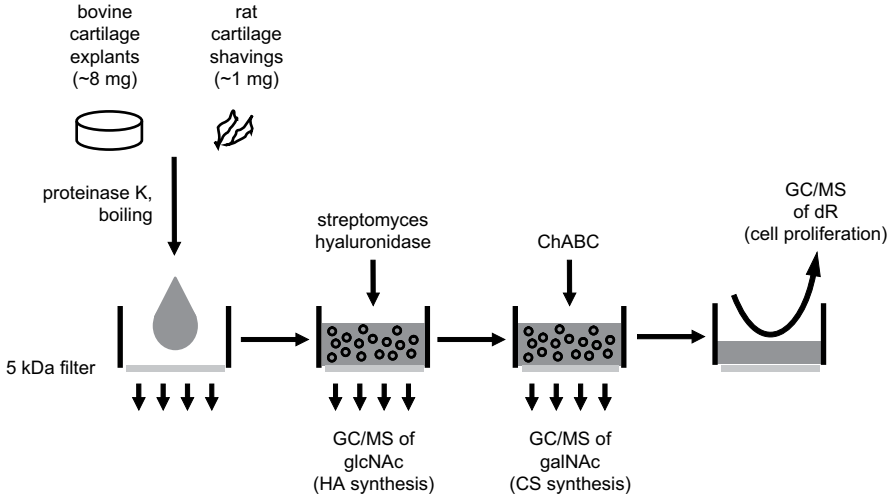

Fig. 1. Protocol for isolating several metabolites from a single specimen of articular cartilage and analyzing by GC/MS. Proteinase K-solubilized cartilage is subjected to sequential digestion steps with hyaluronidase and chondroitinase ABC to separate the molecular constituents of HA, CS, and DNA. Further hydrolysis and derivatization steps (not shown) are used to prepare glcNAc, galNAc, and dR for GC/MS analysis, providing indices of the fractional synthesis HA, CS and DNA, respectively.

deoxyribose (dR) were analyzed by GC/MS as representative analytes of each polymer (CS, HA, and DNA, respectively). GC retention times were established with unlabeled standards, and the abundances of M_0, M_1, and M_2 mass isotopomers were analyzed by selected ion monitoring, using a model 5973 mass spectrometer attached to a 6890 gas chromatograph (Agilent, Palo Alto, CA) and negative chemical ionization (NCI). GC column was DB17 (30 m, 0.25 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA), the helium gas flow rate was adjusted to 1.0 ml/min, and the temperature was programmed, following an initial hold at 140°C for 1 min, to rise from 140 to 300°C at 15°C/min. Isotope enrichments of the M_1 and M_2 mass isotopomers of ^{2}H-labeled samples (EM_1 and EM_2, respectively) were calculated by subtracting the fractional abundances of unlabeled standards.

MEASUREMENT OF ^2H_2O ENRICHMENTS IN CULTURE MEDIUM AND TERMINAL RAT PLASMA

Aliquots of plasma (100 μl), placed into the caps of tightly sealed, inverted screw-capped vials, were kept at 60°C in a heating block overnight. The ^{2}H enrichment of the condensate collected in the vial was measured by reacting with calcium carbide to form acetylene. Acetylene samples were then analyzed using a Series 3000 cycloidal mass spectrometer (Monitor Instruments, Cheswick, PA, USA), modified to record ions at m/z 26 and 27 (M_0 and M_1) and calibrated against a standard curve prepared by mixing 99.9% ^2H_2O with unlabeled water.

CALCULATIONS

In order to interpret label incorporation from ^2H_2O into an end-product, it is necessary to determine the number of hydrogen atoms (n) in the molecule that originated from body H_2O during biosynthesis. While n has been established by use of long-term labeling or combinatorial analysis of mass isotopomer labeling patters for DNA[15] and other metabolic products[15,27–30], it was not known for GAG molecules, and it may vary under different *in vivo* and *in vitro* labeling conditions. Once the number of sites of ^{2}H incorporation has been determined for a molecule, the asymptotic EM_1 value corresponding to 100% newly-synthesized molecules (A_1*) can be calculated for any ^2H_2O concentration, as discussed previously[16,31]. Since the measured EM_1 of a labeled

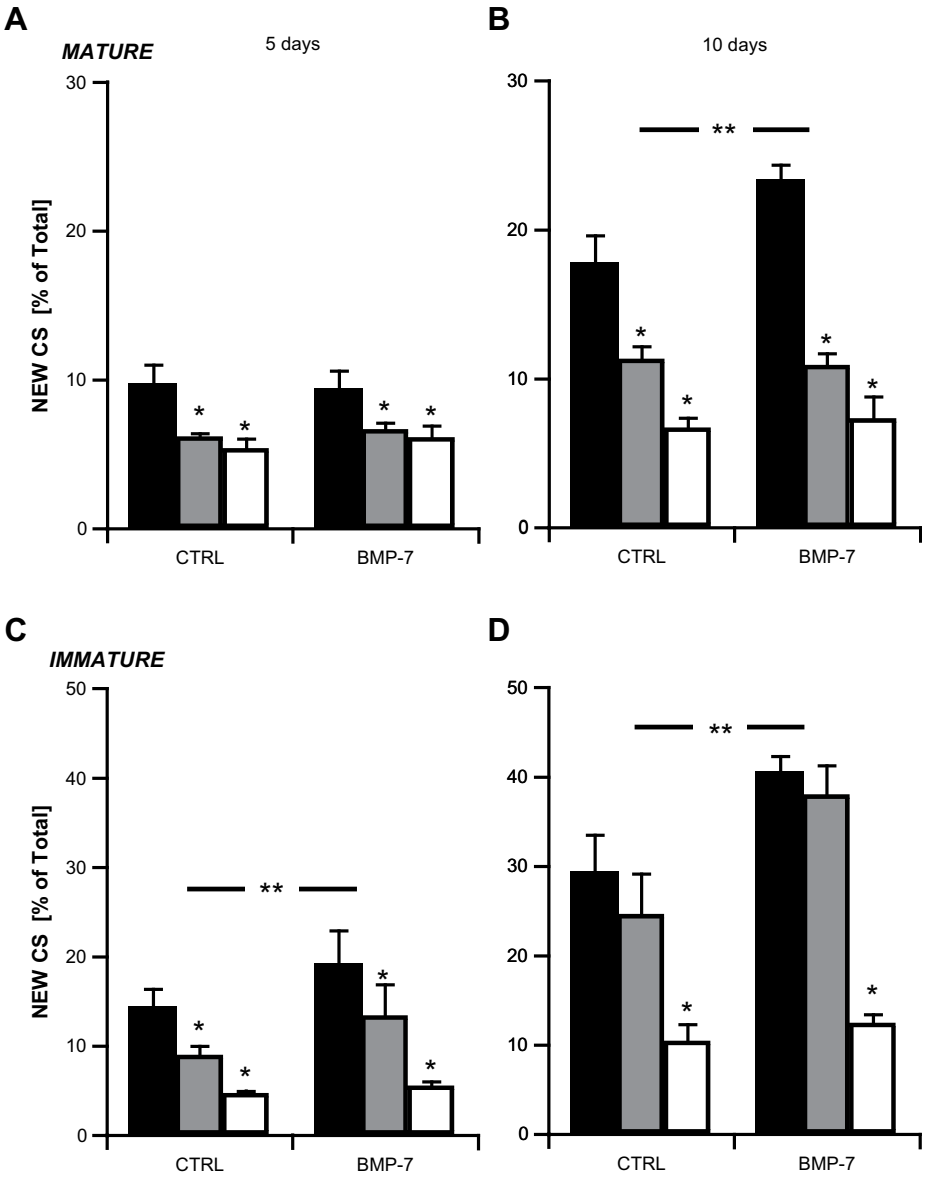

Fig. 2. Age-dependent effects of IL-1 and BMP-7 on the biosynthesis of CS in bovine cartilage explants. Cartilage explants were maintained in medium containing 0 (■), 0.5 (▩), or 5 (□) ng/ml IL-1α, with or without 100 ng/ml BMP-7. Fractional CS synthesis in mature bovine explants after (A) 5 and (B) 10 days in culture, as well as that in immature bovine explants after (C) 5 and (D) 10 days is shown. $^{**}P < 0.05$ for BMP-7 main effect; $^{*}P < 0.05$ compared with IL-1-free controls. $n = 8$ from triplicate experiments.

molecule represents the weighted average of unlabeled (old) and fully labeled (new) molecules, the fraction of molecules that were newly-synthesized (f) can be calculated. Specifically, n for galNAc and glcNAc was determined to 4.3 ± 0.8 in 10 tissue culture experiments and 6.1 ± 0.7 in eight rat experiments (additional details available as online Supplementary material). For ^2H_2O enrichments of 7.2% *in vitro* and 5% *in vivo*, A_1* for newly-synthesized GAG molecules is 16.0 and 16.3%, respectively. Values of f for CS and HA were calculated by dividing the measured EM_1 of galNAc and glcNAc molecules by their asymptotic A_1* value. For fractional DNA synthesis, measured EM_1 in dR was divided by its A_1* value, based on $n = 5$, as established previously[10,15,31]. Absolute synthesis rates were calculated by multiplying f by pool sizes, measured biochemically (see below).

BIOCHEMISTRY

Cartilage digest solutions and spent medium were analyzed for sGAG content after incubation with dimethylmethylene blue (DMB, Polysciences, Warrington, PA)[32], using type C CS from shark cartilage (Sigma–Aldrich, St. Louis, MO) as a standard. DNA content in the cartilage digests was assessed by fluorometry after incubation with PicoGreen® (Molecular Probes, Eugene, OR)[33] and converted to cell number, assuming 7.7 pg DNA/cell[34].

STATISTICAL ANALYSIS

All data are expressed as mean ± standard deviation. Statistical analysis was implemented with Systat 5.2 (Systat, Inc., Evanston, IL, USA). For most comparisons, the main treatment effects (IL-1 or BMP-7) were assessed by one- or two-way analysis of variance (ANOVA), with Tukey *post hoc* testing. In all statistical tests, independent experiments were introduced as a random variable. For rat studies, deuterium incorporation curves were fit to a first-order exponential rise-to-plateau function (SigmaPlot, Systat, Inc., Evanston, IL, USA), and half-lives ($t_{1/2}$) were calculated from the time constant (k) as $t_{1/2} = \ln 2/k$. Cumulative f over the first 14 days in rats also was analyzed by linear regression.

Results

EFFECT OF IL-1 AND BMP-7 ON CS SYNTHESIS IN BOVINE EXPLANTS *IN VITRO*

Adult bovine cartilage explants incubated with serum-supplemented control medium (without IL-1 and BMP-7) maintained a relatively constant sGAG content between 5 and 10 days (350 ± 50 and 330 ± 30 μg/disk, respectively; $P = 0.9$, $n = 8$). Under these steady-state culture conditions, the explants released sGAG into the medium at a rate of 7 ± 2 μg/day, representing an sGAG turnover of 2.2 ± 0.7%/day.

For tissue CS, f was determined from deuterium-labeled galNAc molecules in CS chains. In control studies, the measured fractional CS synthesis did not vary when different enrichments of ^2H_2O were used in the culture medium ($f = 13.2 \pm 1.1$ and 13.9 ± 1.9% after 5 days, for 4 and 8% ^2H_2O, respectively; $P = 0.9$, $n = 4$), confirming that heavy water exposure at these concentrations did not affect cartilage physiology. For adult bovine explants maintained under control conditions, 10 ± 1% of the total CS was newly-synthesized over the first 5 days of serum-supplemented culture [Fig. 2(A)], and f increased over time, reaching 18 ± 2% in the day 10 samples [Fig. 2(B)]. This isotopic result represents an f of 1.9%/day, quantitatively similar to the turnover rate estimated from sGAG release into the medium in the same samples (see above).

To induce catabolic changes in the cartilage explants, cultures were stimulated with IL-1. The inclusion of IL-1 did not detectably alter the sGAG content of adult explants at day 5 ($P = 0.7$), but by day 10, tissue sGAG content was diminished by 27% with 0.5 ng/ml IL-1 ($P = 0.02$) and by 86% with 5 ng/ml ($P \leq 0.001$; data not shown). The addition of BMP-7 at 100 ng/ml had no effect on total sGAG content at either timepoint ($P = 0.8$ and 0.7 for days 5 and 10, respectively).

Heavy water labeling revealed that IL-1 dose-dependently inhibited the synthesis of CS in the adult explants maintained for 5 and 10 days by up to 63%, down to f of 1.2%/day [Fig. 2(A,B); $P < 0.001$ at both timepoints]. The inclusion of 100 ng/ml BMP-7 stimulated fractional CS synthesis by 32% at day 10 (Tukey $P = 0.01$), unless IL-1 was present (interaction $P = 0.03$).

Biosynthesis of CS was also measured in explants of immature, growing articular cartilage, harvested from knee joints of 1–3-week old newborn calves. Like the adult explants, calf explants maintained in basal medium exhibited constant levels of tissue sGAG content between 5 and 10 days of culture (531 ± 42 and 538 ± 74 μg, respectively). In these immature cartilage explants, the cumulative f increased over time, from 14 ± 2% new CS at day 5 to 29 ± 4% at day 10 [Fig. 2(C,D); $n = 8$]. The daily f was 2.9 ± 0.4%/day, which was similar to the rate at which sGAG was released into the medium (2.6 ± 0.9%/day), but 53% higher than that in mature tissue [Fig. 2(A,B); $P < 0.01$]. In contrast, the absolute synthesis rate of sGAG was only 15% higher when expressed on a per cell basis [80 ± 19 and 98 ± 11 pg/day over the first 5 days for adult and calf chondrocytes, respectively; Fig. 3(A,C)]. The highest dose (5 ng/ml) of IL-1 inhibited f by 60–61% in the calf-derived explants labeled for 5 or 10 days [Fig. 2(C,D); $P < 0.01$ at both timepoints], similar to the response of adult cartilage, but inhibition of f by the intermediate dose of IL-1 (0.5 ng/ml) was modest at day 5 (Tukey $P < 0.05$) and undetectable at day 10 [Tukey $P = 0.3$; Fig. 2(D)]. The inclusion of 100 ng/ml BMP-7 stimulated f by an average of 30% (ANOVA $P < 0.05$ for both timepoints) without a statistically significant interaction with IL-1 (interaction $P = 0.13$–0.15) [Fig. 2(C,D)].

EFFECT OF IL-1 AND BMP-7 ON HA SYNTHESIS IN BOVINE EXPLANTS *IN VITRO*

Fractional synthesis of tissue HA was determined by [^{2}H]-label incorporation into glcNAc molecules in HA chains. For HA, f was 1.3 ± 0.2%/day in adult cartilage explants maintained under control conditions, 32% slower than f for CS. HA synthesis increased by an average of 32% ($P = 0.03$) by addition of IL-1 at the early timepoint [Fig. 4(A)], but by day 10, the stimulation had dissipated [Fig. 4(B); $P = 0.6$]. BMP-7 had no effect on HA synthesis at either timepoint ($P = 0.7$–0.8).

In contrast, in immature bovine cartilage, f for HA was 2.3 times higher than in the adult cartilage [~3%/day; Fig. 4(C,D)], but regulation by IL-1 and BMP-7 more closely resembled the CS response, in that IL-1 was markedly inhibitory ($P = 0.02$ at both timepoints) and BMP-7 was markedly stimulatory ($P = 0.02$–0.03).

EFFECT OF IL-1 AND BMP-7 ON DNA SYNTHESIS IN BOVINE EXPLANTS *IN VITRO*

In adult cartilage explants, chondrocyte proliferation was generally low, with 0.2–0.4% of the total cell population proliferating per day. This low basal level of cell turnover was not detectably affected by either BMP-7 or IL-1 [Fig. 5(A,B); $P = 0.2$–0.4].

In contrast, cell proliferation in newborn cartilage exhibited a temporal lag, with the average f for DNA initially similar to that in the adult [0.55 ± 0.25%/day; $n = 8$; Fig. 5(C)], but increasing 6.5-fold between days 5 and 10 to an average of 2%/day [Fig. 5(D)]. Proliferation was inhibited by IL-1 at both timepoints ($P = 0.03$ on day 5 and $P < 0.001$ on day 10) and there was an overall 2-fold stimulation by BMP-7 ($P = 0.02$ on day 5 and $P < 0.001$ on day 10), except at the highest IL-1 dose (interaction $P = 0.001$ on day 10).

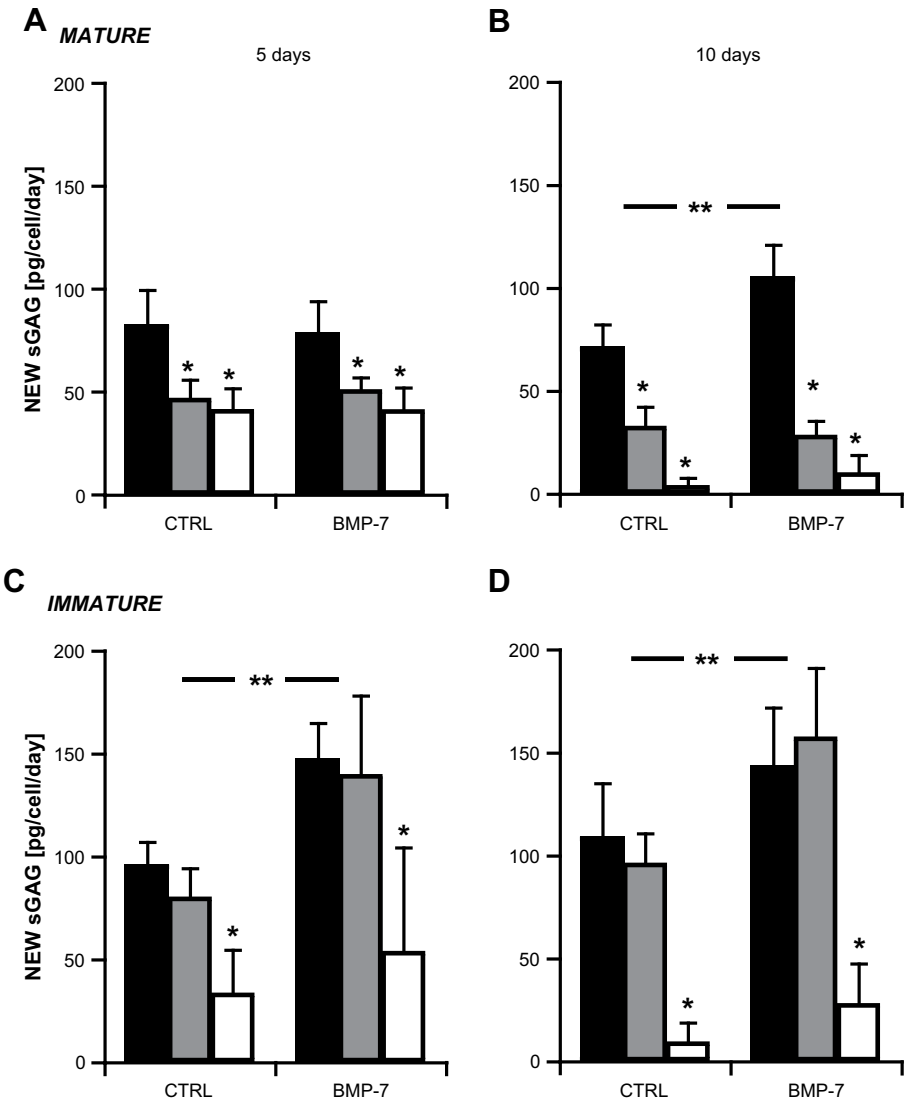

Fig. 3. Age-dependent effects of IL-1 and BMP-7 on the biosynthesis rate of sGAG in bovine cartilage explants, expressed as sGAG synthesized per day. Cartilage explants were maintained in medium containing 0 (■), 0.5 (▩), or 5 (□) ng/ml IL-1α, with or without 100 ng/ml BMP-7. Absolute CS deposited is determined from the product of fractional CS synthesis and total tissue content of sGAG and presented normalized to cell number. CS biosynthesis was determined for mature bovine explants maintained for (A) 5 and (B) 10 days and for immature bovine explants maintained for (C) 5 and (D) 10 days. $^{**}P < 0.05$ for BMP-7 main effect; $^{*}P < 0.05$ compared with IL-1-free controls. $n = 8$ from triplicate experiments.

IN *VIVO* MEASUREMENTS OF CS SYNTHESIS AND CELL PROLIFERATION IN ARTICULAR CARTILAGE

After receiving ^2H_2O, the average body water enrichment of rats sacrificed at timepoints ranging from 2 to 365 days was $5.2 \pm 0.5\%$ ($n = 50$). Based on the measured value of $n = 6$ C–H bonds (see online Supplementary material), this ^2H_2O enrichment (P) corresponds to an asymptotic EM1 (A_1*) of 16.3% for newly-synthesized galNAc in CS. After 365 days of ^2H_2O, enrichment of CS from the cartilage of the MTP reached $95 \pm 0.2\%$ of this asymptote, confirming the calculated theoretical maximum enrichment and indicating virtually complete CS turnover (Fig. 6). Over the initial 17 days of ^2H_2O labeling, fractional CS synthesis in the rat MTP appeared to be relatively linear ($r^2 = 0.94$, $P < 0.01$, $n = 48$), averaging $2.6 \pm 0.5\%$/day, similar to the turnover rate of CS seen in adult and immature bovine cartilage *in vitro* over 10 days in culture. Overall, the kinetics of CS in the rat MTP exhibited a first-order half-life of 22 days.

In contrast to the CS turnover rate in articular cartilage of the rat, chondrocyte proliferation was only $44 \pm 4\%$ after 1 year ($t_{1/2} = 862$ days), and the initial rate of cell proliferation ($0.68 \pm 0.09\%$/day; $n = 2$) was comparable to values in the explants from newborn calves at the onset of culture. Of note, articular cartilage *in vitro* in rats and from newborn calf explants both exhibited detectable cell proliferation, unlike adult bovine explants.

Discussion

We describe here a method for direct measurement of the synthesis rates of multiple metabolites simultaneously from a single specimen of cartilage that is also applicable *in vivo*.

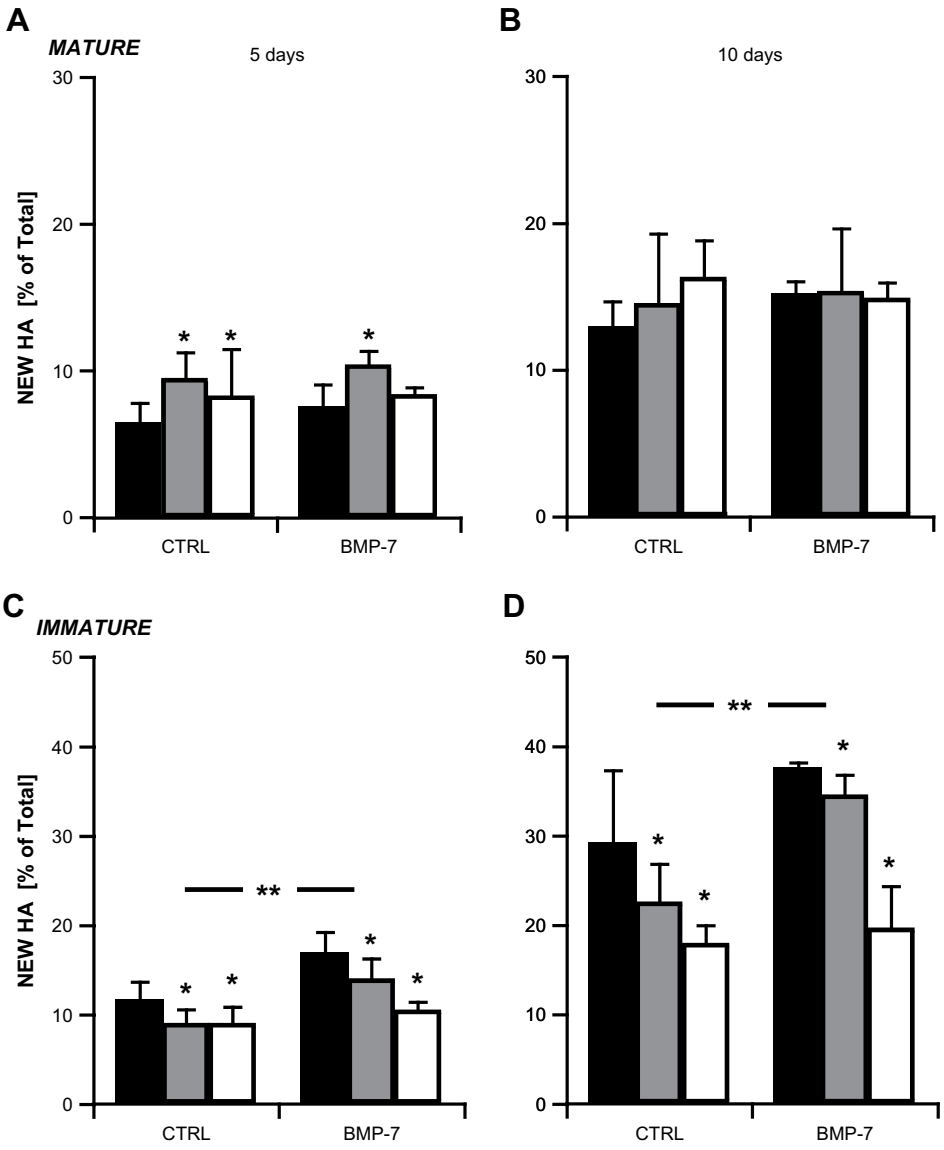

Fig. 4. Age-dependent effects of IL-1 and BMP-7 on the biosynthesis of HA in bovine cartilage explants. Cartilage explants were maintained in medium containing 0 (■), 0.5 (▨), or 5 (□) ng/ml IL-1α, with or without 100 ng/ml BMP-7. Fractional HA synthesis in mature bovine explants after (A) 5 and (B) 10 days in culture, as well as that in immature bovine explants after (C) 5 and (D) 10 days is shown. $^{**}P < 0.05$ for BMP-7 main effect; $^{*}P < 0.05$ compared with IL-1-free controls. $n = 8$ from triplicate experiments.

The data presented here validate in several ways the stable isotope-mass spectrometric techniques for monitoring articular biology *in vivo*. First, a widely-used *in vitro* model system was characterized kinetically, followed by the *in vivo* application of the method over an extended period of time. The similarities between isotopically measured CS synthesis rates and CS release in steady-state culture of cartilage explants and the finding that rat cartilage-derived CS was enriched with deuterium to 95% of the theoretical maximum value after a year on heavy water both support the quantitative nature of this method.

Matrix and cell turnover in articular cartilage were quantified after the administration of $^{2}H_2O$ from the labeling of glcNAc, galNAc and dR derived from HA, CS and DNA, respectively. Sufficient yields of the latter two analytes were obtained from a single specimen of cartilage at least 0.3 mg (by wet weight); ca. 1 mg of cartilage was necessary to reproducibly analyze HA. While the enzyme-based methods used to isolate these metabolites do not achieve purity, the subsequent GC/MS analysis (which involves both GC separation and mass identification) ensured the purity of the analytes. The degree to which cultured cartilage explants incorporated ^{2}H into newly-synthesized HA, CS, and DNA was quantified, enabling measurements of matrix synthesis and cell proliferation that were modulated positively by BMP-7 and negatively by IL-1.

IL-1 is a pro-inflammatory cytokine that contributes to the disruption of matrix homeostasis in joint diseases[35], in part by exerting inhibitory effects on aggrecan gene expression[36] and the deposition of newly-synthesized sGAG[6,20,22,37]. In bovine explant cultures, we show that IL-1 inhibits the fractional synthesis of CS in both adult and immature cartilage by as much as 63% at a concentration of 5 ng/ml. This result is quantitatively similar to previous studies on cartilage metabolism, in which bovine

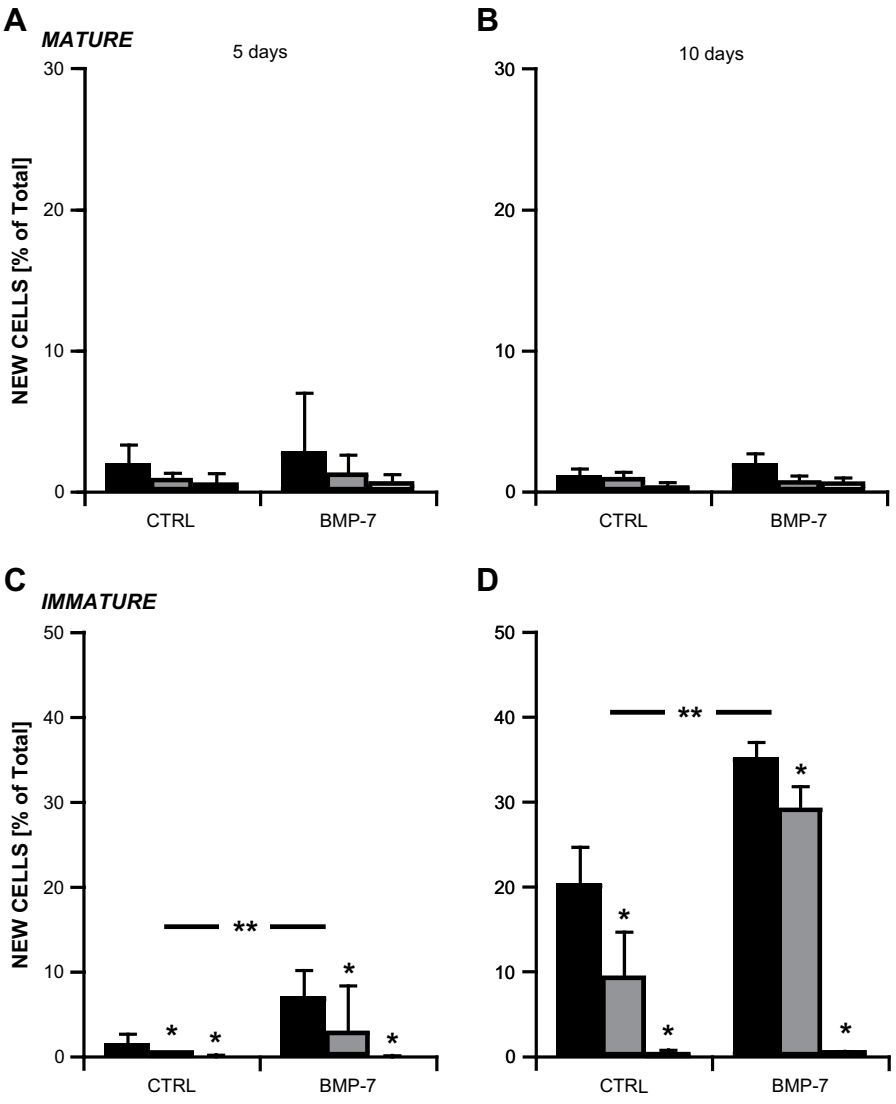

Fig. 5. Age-dependent effects of IL-1 and BMP-7 on cell proliferation in bovine cartilage explants. Cartilage explants were maintained in medium containing 0 (■), 0.5 (▩), or 5 (□) ng/ml IL-1α, with or without 100 ng/ml BMP-7. Fractional DNA synthesis in mature bovine explants after (A) 5 and (B) 10 days in culture, as well as that in immature bovine explants after (C) 5 and (D) 10 days is shown. $^{**}P < 0.05$ for BMP-7 main effect; $^{*}P < 0.05$ compared with IL-1-free controls. $n = 8$ from triplicate experiments.

cartilage maintained under similar culture conditions exhibited a 50–65% inhibition in radiosulfate incorporation when exposed to such high doses of IL-1[21,38]. Furthermore, we found that under the serum-supplemented conditions used here, 0.5 ng/ml IL-1 inhibited fractional CS synthesis in mature, but not immature, bovine cartilage, indicating higher sensitivity of mature cartilage to degenerative changes. This is consistent with *in vivo* studies in mice[39,40] and rabbits[37] but other *in vitro* studies that directly compare the inhibition of GAG by IL-1 on young and old cartilage have shown mixed results[40–43], perhaps due to differences in culture conditions. Indeed, van Beuningen and co-authors reported that age-related differences in IL-1 sensitivity dissipated when using defined, rather than serum-supplemented, medium[40], a finding that underscores that the counteracting response to anabolic growth factors modulates the effect of IL-1. In our study, IL-1 also suppressed the rates of fractional HA synthesis and cell proliferation in immature bovine cartilage, whereas adult cartilage exhibited a moderate upregulation of HA synthesis in response to IL-1, which has been demonstrated previously for chondrocytes isolated from bovine animals[44,45]. In immature cartilage especially, the stimulation of DNA and GAG synthesis by BMP-7 was sufficient to offset the inhibitory effects of IL-1, consistent with the chondroprotective role of BMP-7 shown *in vitro*[46] and in animal studies[47]. Although BMP-7 had been thought to exert negligible effects on chondrocyte proliferation[7], our data indicate that BMP-7, in combination with serum, induced a marked stimulation of DNA synthesis in immature chondrocytes. This finding extends a recent report in which the proliferation of adult chondrocytes, isolated from their native cartilage matrix, was enhanced by the synergistic effects of BMP-7 combined with insulin-like growth factor-1[48]. Age-dependent variations in the response to regulatory factors such as IL-1, BMP-7 and those found in serum, as demonstrated here and elsewhere[6], may clarify why young cartilage is less

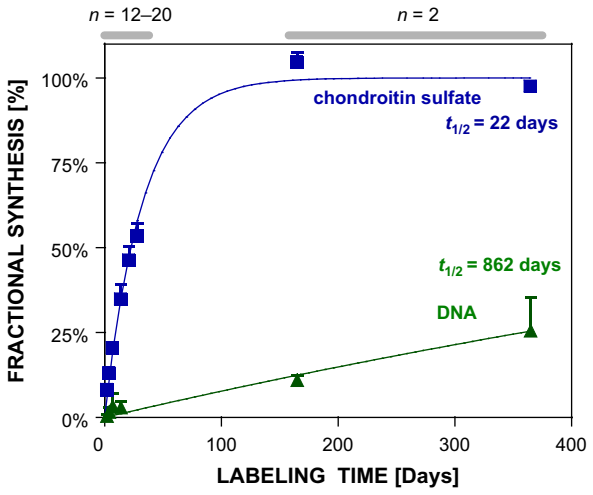

Fig. 6. Fractional synthesis of CS & DNA (cells) *in vivo* in rat knee cartilage.

susceptible to the degenerative changes associated with OA. Of interest, we found that the fractional synthesis rates of CS were 53% higher in calf cartilage than in adult cartilage, but that the absolute synthesis rate was only 15% higher when expressed on a per cell basis, suggesting that reduced cellularity at later stages of growth accounts for the majority of the age-related decline.

Stable isotope-mass spectrometric approaches are particularly well-suited for kinetic studies that require *in vivo* measurements. In 1952, 16-day pulse-chase studies using adult rats estimated the half-life of incorporated radiosulfate to be 17 days in rib cartilage[49], comparable to the physiological turnover rate of articular cartilage CS reported here ($t_{1/2} = 22$ days). More recently, *ex vivo* tissue culture models have been more commonly used. Maroudas and coworkers demonstrated in rabbits and dogs that *in vitro* labeling of freshly harvested cartilage corresponded with *in vivo* labeling[50], suggesting that under controlled conditions, it is possible to obtain a quantitative measure of sGAG synthesis on the basis of *ex vivo* experiments. When interpreting data from such assays *in vitro*, however, it is important to consider the injurious effects of tissue lacerations on the viability of adjacent cells[51], the diffusivity of the tracer in short-term labeling protocols, and the presence or absence of confounding medium components, such as serum. Moreover, OA, in particular, is a disease that involves interaction between multiple tissues, and as such, many relevant aspects of the pathology are difficult to reproduce *in vitro*.

Measurement of cartilage-related kinetics using heavy water allows for *in vivo* metabolic measurements over a range of labeling intervals. The incorporation of deuterium into newly-synthesized CS in rat articular cartilage was approximately linear for the first 2 weeks when heavy water was administered, with cumulative values of f reaching $35 \pm 4\%$ after 14 days. On the other hand, mitosis occurred *in vivo* on a much slower timescale, with only 44% of the chondrocytes labeled as new after 1 year. We began labeling in rats at an age that is considered skeletally mature[52]. In rats[53] and in other skeletally-mature animal models[54–56], chondrocyte proliferation has been reported to be a rare event when tritiated thymidine was used as a label. Since those studies involved short-term labeling periods (e.g., 4 h[56]), low labeling indices on the order of one mitotic cell per 1000 would result if only 0.68% of chondrocytes turnover per day, as we measured, which may be difficult to detect with autoradiography. The ability of heavy water labeling techniques to make metabolic measurements over an extended period of time is a potential advantage for monitoring the effects of regulatory factors that are expected to exert their influence over long periods of time, such as the cumulative, time-averaged effects of exercise (joint loading), the chronic effects of OA, or the *in vivo* pharmacodynamics of therapeutic interventions.

This kinetic approach has potential clinical applications. Heavy water has been studied in animal models as well as humans for over 60 years. Extensive studies with long-term administration in animal models indicate that there are no adverse effects on normal physiology, including growth, appetite, reproduction, neoplasia or hematological parameters, at ^2H_2O levels several-fold higher than in the present studies[57,58]. Indeed, at levels of deuterium enrichment in body water below 15%, harmful effects in mammals have not been detected[58,59]. Moreover, maintenance of $>$15% deuterium enrichment in body water requires ongoing intake of drinking water at even higher enrichments (20–25% or higher). These enrichments of body water or drinking water are several-fold higher than those in the present studies, or in any human studies carried out with this method. Heavy water has also been given alone[10,16,18,60–63], or as part of doubly labeled water protocols for measuring energy expenditure[64–68], to thousands of human subjects without observable adverse effects. The heavy water labeling approach described here has been successfully applied in preliminary studies of non-load-bearing articular cartilage sampled in humans at the time of routine notchplasty surgery, following a few weeks of oral heavy water intake (unpublished observations). Moreover, it may be possible to develop synovial fluid biomarkers of cartilage metabolism following heavy water intake, raising the prospect of using less invasive methods.

Conflict of interest

Some authors (KWL, SAS, EWC, MA, SMT) were full-time employees of KineMed, Inc. while contributing to this research. MKH is Chief of the Scientific Advisory Board of KineMed, Inc. and has received consulting income and stock considerations.

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Supplementary material

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