Prolonged Treatment of Human Osteoarthritic Chondrocytes with Insulin-like Growth Factor-I Stimulates Proteoglycan Synthesis But Not Proteoglycan Matrix Accumulation in Alginate Cultures

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ABSTRACT. ***Objective***. To determine the level of anabolic response when chondrocytes isolated from human osteoarthritic cartilage are stimulated with 2 doses of insulin-like growth factor-I (IGF-I) for extended culture periods.

Methods. Human chondrocytes were isolated from knee cartilage removed at the time of joint replacement surgery for osteoarthritis (OA). The cells were cultured in alginate beads under serum-free conditions and treated with 100 ng/ml or 1000 ng/ml of human recombinant IGF-I. Response was measured during culture periods of 1 to 28 days by determining the level of radiolabeled sulfate incorporated into alcian blue precipitable material and by measuring the level of total proteoglycan accumulation using the dimethylmethylene blue (DMB) assay. For the latter assay, cultures treated with osteogenic protein-1 (OP-1) were used for comparison to IGF-I. Results were normalized to cell numbers using DNA measurements.

Results. The level of IGF-I stimulated sulfate incorporation relative to untreated controls increased with time in culture, with a peak response occurring between days 7 and 14 of culture. There was no significant difference between the 2 IGF-I doses. Despite the stimulation of sulfate incorporation, the DMB assay did not reveal a significant accumulation of proteoglycans in the cell-associated and further-removed matrix with either dose of IGF-I in cultures carried out to 21 days. In contrast, compared to controls, OP-1 at 100 ng/ml stimulated a 3-fold increase in matrix proteoglycan at day 21 of culture.

Conclusion. Prolonged IGF-I treatment of human OAchondrocytes in serum-free alginate cultures stimulated sulfate incorporation without significant accumulation of a proteoglycan matrix in longterm cultures. However, significant proteoglycan accumulation was seen in cultures treated with OP-1, suggesting it is a better stimulator of proteoglycan production by OA chondrocytes. (J Rheumatol 2003;30:1565–70)

Key Indexing Terms:
OSTEOARTHRITIS CHONDROCYTE INSULIN-LIKE GROWTH FACTOR-I
OSTEOGENIC PROTEIN-1 CARTILAGE REPAIR

Insulin-like growth factor-I (IGF-I), which is found in both normal and osteoarthritic articular cartilage, has been felt to be a key growth factor capable of autocrine and paracrine regulation of chondrocyte proteoglycan synthesis[1-6]. Much of the information regarding the ability of IGF-I to stimulate chondrocyte proteoglycan as well as collagen production has come from *in vitro* studies, where cartilage explants or isolated chondrocytes from normal cartilage have been treated with exogenous IGF-I[7-10]. In addition to stimulation of matrix synthesis, IGF-I stimulates chondrocyte integrin expression[11], and autocrine production of IGF-I helps to promote chondrocyte survival[12]. Because of these activities, IGF-I has been considered to be a potential therapeutic agent for conditions resulting from cartilage lesions or cartilage matrix degradation, including that resulting from osteoarthritis (OA)[13,14].

To judge the feasibility of using IGF-I therapeutically in conditions such as OA, it is important to know how well OA chondrocytes respond to IGF-I. The few studies that have

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examined the ability of IGF-I to stimulate chondrocytes obtained from arthritic cartilage have not been able to demonstrate a significant anabolic response. These studies have some limitations in their applicability to human OA chondrocytes in that they only tested doses of IGF-I up to 15 ng/ml[15], only tested monolayer cultured cells[16], or tested cells from a mouse inflammatory arthritis model[17].

The goal of our study was to measure the response of human OA chondrocytes to IGF-I in both short-term and longterm cultures, the latter of which would enable the assessment of matrix production. The cells were cultured in suspension in alginate beads in order to maintain the differentiated chondrocyte phenotype[18], which can be lost with time in monolayer culture[19,20]. Additional advantages of the alginate system are that chondrocytes in alginate can produce a matrix similar to native cartilage, and the alginate system can be used to measure the proteoglycan-containing matrix produced and retained by the cells[21]. The amount of proteoglycan produced and deposited in the matrix after IGF-I treatment was compared to osteogenic protein-1 (OP-1) since previous studies had shown that OP-1 was a potent inducer of chondrocyte proteoglycan production, although only previously tested in cells from normal cartilage[22].

MATERIALS AND METHODS

Chondrocyte isolation and culture. Articular cartilage was removed from knee joint tissues obtained during joint replacement surgery for OA. A scalpel was used to dissect the remaining cartilage from joint surfaces, with care taken to avoid the underlying bone and osteophytes. Chondrocytes were isolated by enzymatic digestion using 0.2% Pronase (Calbiochem, San Diego, CA, USA) in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1) for 1 h, followed by overnight incubation with 0.025% Collagenase-P (Boehringer-Mannheim, Chicago, IL, USA) in DMEM/Ham's F12 supplemented with 5% fetal bovine serum. After filtering and washing with serum-free medium the viable cells were counted using trypan blue. Only digests with greater than 90% initial viability were used for culture. The yield ranged from 10 to 30 million cells per subject. Cells from each subject were cultured individually. Isolated cells were resuspended at 2×10^6 cells/ml in sodium alginate for making alginate beads as described[23]. The beads were transferred to 24 well plates (4 beads/well) and cultured in 0.5 ml/well of serum-free DMEM:Ham's F-12 (1:1) supplemented with 1% mini-ITS+ containing 5 nM insulin ("mini"-dose insulin so that the IGF-I receptor is not stimulated), 2 µg/ml transferrin, 2 ng/ml selenous acid, 25 µg/ml ascorbic acid, and bovine serum albumin/linoleic acid at 420/2.1 µg/ml[23]. The serum-free media with 1% mini-ITS+ did not contain detectable levels of IGF-I as tested using an IGF-I ELISA kit (Diagnostic Systems Laboratories, Webster, TX, USA), which has a minimal detection limit of 0.01 ng/ml.

In vitro stimulation and measurement of sulfate incorporation. Cells were cultured for 3 days to allow recovery from the isolation procedure and then the medium was exchanged for fresh serum-free mini-ITS+ (control) or serum-free mini-ITS+ medium supplemented with either 100 ng/ml or 1000 ng/ml recombinant human IGF-I (Chiron Corporation, Emeryville, CA, USA). Thereafter, medium was changed every 48 h with the addition of fresh IGF-I. For measurement of sulfate incorporation, 50 µCi/ml of $^{35}SO_4$ (ICN, Costa Mesa, CA, USA) was added to wells for the final 18 h culture period for measurement at each time point. The media were collected and the alginate beads were dissolved in sodium citrate followed by centrifugation to obtain a cell pellet. The supernatant fraction from the dissolved alginate beads was considered to be the further-removed matrix (FRM) and the cell pellet to be the cell-associated matrix (CM) as described[24]. Sulfate incorporation was measured in the media, FRM, and CM fractions using alcian blue precipitation as described in detail[23]. This method has been shown to quantitate sulfated glycosaminoglycan chains of various sizes, including those resulting from papain digestion, and is linear from 2 to 800 µg/ml of proteoglycan[25]. Total DNAwas measured in the cell pellet and used to normalize the sulfate incorporation results as described[23].

Dimethylmethylene blue (DMB) assay for proteoglycan production. Samples of media, alginate matrix, and cell pellets were prepared as for sulfate incorporation but using alginate beads cultured at 8 beads per well in 24 well plates. In addition to the IGF-I treatment groups, a group was treated with 100 ng/ml of recombinant human OP-1 (provided by Stryker Biotech, Hopkinton, MA, USA). Cell pellets were treated with 0.02% sodium dodecyl sulfate followed by digestion with Proteinase K (125 µg/ml; Calbiochem) prior to the DMB assay and DNAanalysis. The DMB assay was performed as described[21] using bovine nasal septum D1 proteoglycan standard (provided by Dr. E. Thonar, Rush Medical College). The DNAassay used PicoGreen (Molecular Probes, Eugene, OR, USA) instead of Hoechst dye.

Statistical analysis. The means and standard errors were calculated and the results analyzed using analysis of variance (ANOVA). The analysis was performed with StatView 5.0 software (SAS Institute, Cary, NC, USA)

RESULTS

Chondrocytes were isolated from knee cartilage obtained from a total of 9 subjects (8 women, one man) with severe OA requiring joint replacement. The subjects ranged in age from 50 to 91 years (mean 69 ± 4.3). Alginate bead cultures from the first 3 subjects were incubated in serum-free control medium, medium containing 100 ng/ml IGF-I, or medium with 1000 ng/ml IGF-I. Sulfate incorporation was measured at days 1, 3, 7, 10, 14, 21, and 28 of treatment. In the cultures from these first 3 subjects, sulfate incorporation was only measured in the media and alginate fractions, while the cell pellet was used for DNA analysis. After one day of IGF-I treatment, sulfate incorporation (combined media and alginate fractions) was only 128% of control with the 100 ng/ml dose and equal to control with the 1000 ng/ml dose (Figure 1). With time in culture, however, the response to IGF-I increased, with a peak for the 100 ng/ml cultures of 172 ± 27% of control at day 14 of treatment, and a peak for the 1000 ng/ml IGF-I group of 168 ± 12% at day 10. By day 28 of continuous IGF-I treatment, sulfate incorporation declined to control levels.

Based on the results of this experiment, cells from the next 3 subjects were treated for 10 days with both doses of IGF-I, and sulfate incorporation was measured at days 3, 7, and 10. Previous studies of chondrocytes cultured in alginate had shown that newly synthesized proteoglycans can be found in 2 matrix compartments: the cell-associated matrix (CM) and the further removed matrix (FRM)[21,24]. Therefore, in this set of experiments, sulfate incorporation was measured in each of the 3 fractions (media, FRM, and CM). To determine if the 10 days of IGF-I treatment had a sustained effect, cultures from the same subjects treated with IGF-I for 10 days were continued in serum-free medium without IGF-I for an additional culture period of 4 and 11 days (days 14 and 21 in Figure 2). Sulfate incorpo-

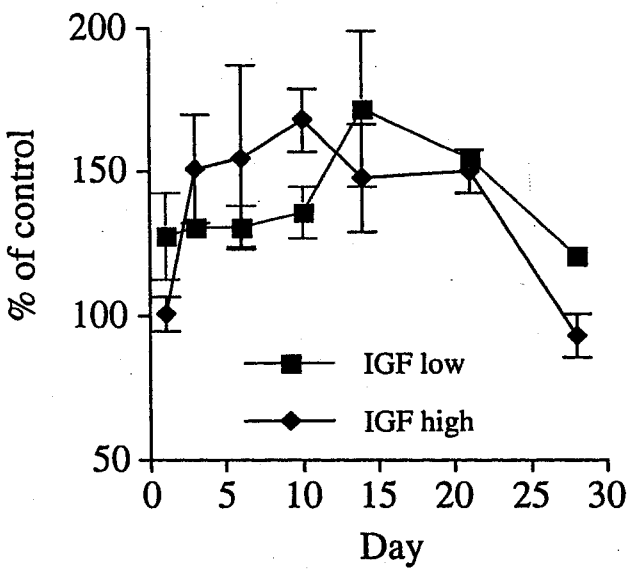


Figure 1. Response of chondrocytes from human OAcartilage to insulin-like growth factor-I (IGF-I) during 28 days of culture. Chondrocytes isolated from 3 subjects were cultured in alginate beads in serum-free control media or media containing 100 ng/ml IGF-I (IGF low) or 1000 ng/ml IGF-I (IGF high). Duplicate wells were analyzed for each subject at each time point. Sulfate incorporation was normalized to cell numbers by DNA analysis. The results of IGF-I treated wells were expressed as a percentage of control wells and represent the mean and SEM for the 3 subjects at each time point.

ration was significantly greater versus controls at days 3 and 7 of culture in samples treated with either dose of IGF-I (Figure 2). Sulfate incorporation declined by day 10, but then appeared to increase at day 14 (although not statistically greater than control due to variability between samples), followed by a decline to control levels by day 21. The results were similar whether sulfate incorporation was analyzed in only the 2 matrix compartments (Figure 2B) or if the medium fraction was also included (Figure 2A).

The distribution of the radiolabel incorporation into the 3 fractions is shown in Figure 2C for day 7 of culture, when the peak IGF effect over control was noted. About 25% of the label was in the cell-associated fraction. Compared to controls, addition of IGF-I stimulated sulfate incorporation in both the FRM and CM. The percentage of the total sulfate incorporation in the matrix fractions increased with IGF-I treatment from 64% in the controls to 72% in cultures treated with 100 ng/ml IGF-I and 78% with 1000 ng/ml IGF-I.

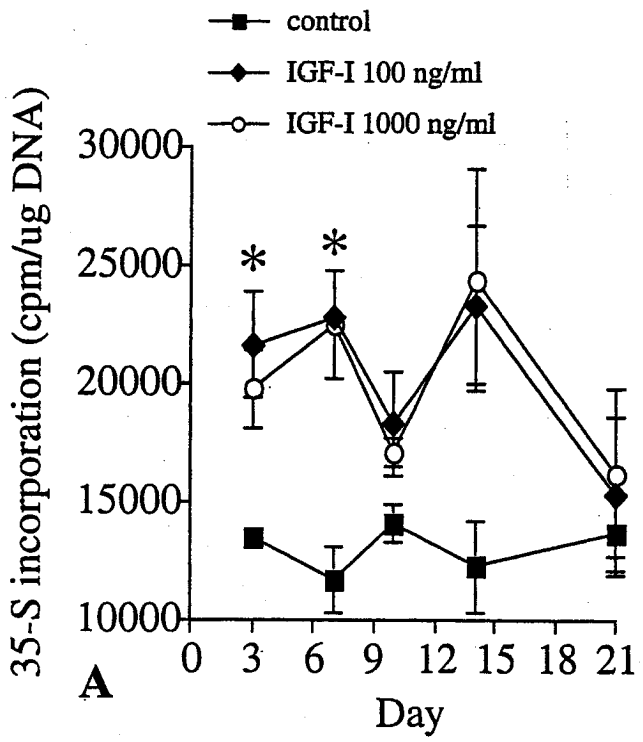
To determine if the observed IGF-I stimulation of sulfate incorporation resulted in significant amounts of proteoglycan being deposited and retained in the chondrocyte matrix, the DMB assay was used to measure total proteoglycan in the cell-associated and alginate matrix in cultures from 3 additional subjects all treated for 3, 7, and 21 days with both IGF-I doses. In these experiments, OP-1 was included for comparison to IGF-I. The DMB assay did not reveal a significant difference from control at any time point with either dose of IGF-I, while treatment with OP-1 at 100 ng/ml resulted in a significant 3-fold increase at day 21 (Figure 3).

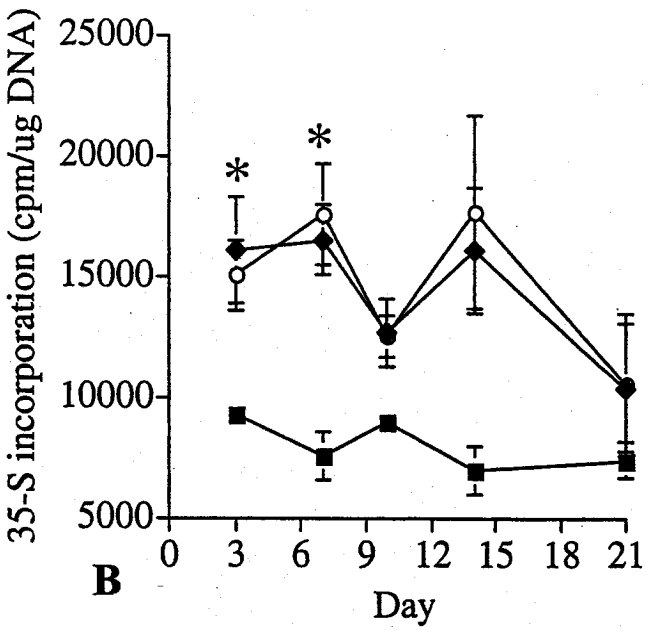
DISCUSSION

Our results confirm and extend previous findings from studies of human OA chondrocytes in explant culture[15,26] and monolayer culture[16], which suggested that chondrocytes from OA cartilage respond poorly to exogenous IGF-I. In our study, culturing the cells in suspension and including a 10-fold higher dose of IGF-I did not stimulate significant sulfate incorporation over controls after 24 hours of treatment. However, prolonged treatment with IGF-I did result in increased sulfate incorporation measured after 3 and 7 days of culture. Despite the increase in IGF-I stimulated sulfate incorporation, an increase in total proteoglycan deposited and retained in the matrix was not observed. In contrast to IGF-I, OP-1 was able to stimulate a 3-fold increase in matrix proteoglycan accumulation, indicating that under the conditions tested OP-1 was a more potent stimulator of OAchondrocyte proteoglycan production.

The sulfate incorporation and DMB assays are standard methods used to measure proteoglycan production. Previous studies have shown that radiolabeled sulfate is almost exclusively incorporated into proteoglycans by chondrocytes and that by precipitating the labeled samples with alcian blue, glycosaminoglycans down to at least 2 µg/ml will be detected[25]. An increase in sulfate incorporation reflects proteoglycan synthesis but does not distinguish the size of the proteoglycan made. The DMB assay can also measure proteoglycans down to 2 µg/ml, although it appears to be more sensitive in detecting large proteoglycans such as aggrecan extracted from cartilage when compared to the small proteoglycans made by other tissues[27]. As used in our study, the sulfate incorporation assay measured proteoglycan synthesis at the time of the radiolabel pulse, while the DMB assay at day 21 measured the total amount of proteoglycan produced and retained in the matrix (FRM plus CM). Therefore, the finding that IGF-I stimulated sulfate incorporation without an accompanying increase in proteoglycan accumulation at day 21 suggests that IGF-I stimulated sulfate incorporation was primarily in small proteoglycans that were not retained over time in the matrix and/or not detected as well by the DMB assay. Previous work in the alignate system showed that, unlike aggrecan, small proteoglycans were not retained very well in the matrix[21,24].

The other possible explanation for a lack of accumulation of proteoglycan in IGF-I treated cultures would be that the proteoglycans were degraded by endogenous enzymes produced by the OA chondrocytes[28]. This possibility would be consistent with a reported finding that intraarticular





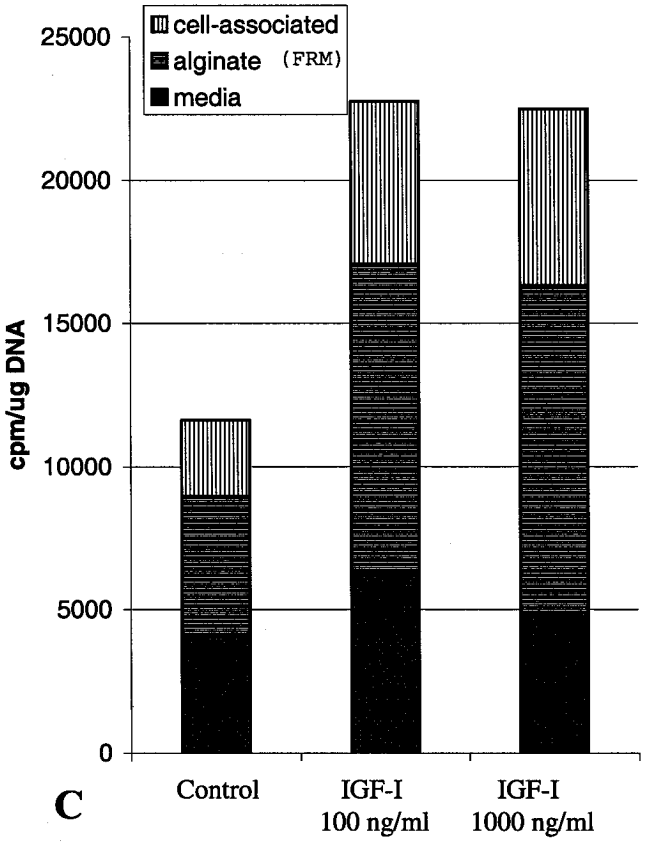


Figure 2. Insulin-like growth factor-I (IGF-I) stimulation of human OA chondrocytes for 10 days followed by IGF-I withdrawal. Chondrocytes from 3 subjects with OAwere each treated separately, as detailed in Figure 1, except IGF-I treatment was stopped at day 10 of culture, and sulfate incorporation was measured in 3 fractions: media, alginate matrix or further-removed matrix (FRM), and cell-associated matrix (CM). A. Total sulfate incorporation into all 3 fractions. B. Sulfate incorporation into FRM plus CM fractions. C. Distribution of sulfate label into the 3 fractions at culture day 7. *p < 0.04 control vs IGF-I by ANOVA.

injections of IGF-I in the anterior cruciate ligament-deficient canine OAmodel did not result in significant improvement in histological scores when given alone, but did when combined with sodium pentosan polysulfate, which was thought to inhibit proteinase activity[29]. Similarly, although expression of IGF-I in rabbit joints by gene transfer was able to stimulate proteoglycan synthesis in an antigen-induced arthritis model, IGF-I did not have a protective effect on the release of proteoglycans from the cartilage[30].

Because the cells tested in our study were isolated from older adults with severe OA, a reduced anabolic response to IGF-I, due to changes associated with both aging and OA, may explain why endogenous catabolic pathways could predominate over anabolic pathways. An age-related reduction in the IGF-I anabolic response has been noted in animals, including rats[31,32], cows,[33] and monkeys[23]. A reduced IGF-I anabolic response has also been noted in an inflammatory arthritis model in mice[17] and in osteoarthritic-like cartilage from monkeys[23]. The mechanism responsible for the reduced IGF-I response is not clear and may differ between normal cartilage aging and changes occurring in OA cartilage.

In OA cartilage, an increase in IGF binding protein (IGFBP) production has been noted and has been hypothesized to be responsible for reducing the IGF response[16,34,35]. By binding IGF-I, and thereby competing for IGF receptor

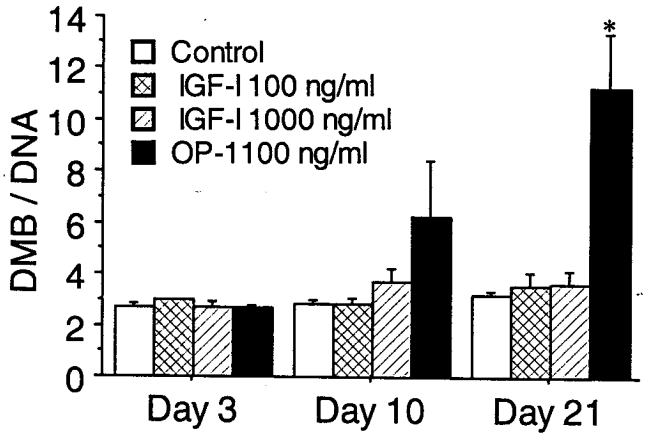


Figure 3. Effects of insulin-like growth factor-I (IGF-I) and osteogenic protein-1 (OP-1) on proteoglycan deposition by human OAchondrocytes. Chondrocytes from 3 subjects were cultured for 3, 10, and 21 days in serum-free control media (control) or serum-free media supplemented with 100 ng/ml IGF-I, 1000 ng/ml IGF-I, or 100 ng/ml OP-1. The DMB assay was used to measure proteoglycan levels in the further-removed matrix and the cell-associated matrix and normalized to cell numbers by DNA analysis. *p = 0.0002 control vs OP-1 by ANOVA.

binding, the IGFBP can inhibit IGF action, while under certain conditions they can promote the actions of IGF by transporting IGF to receptor sites or by concentrating and storing IGF-I in the pericellular space, where it can be released to stimulate the receptor (reviewed in[36,37]). In our study we increased the dose of IGF-I to 10-fold the dose used in previous studies in an effort to use levels of IGF-I that might overwhelm the capacity of the IGFBP. The finding that the cells did not respond to the 1000 ng/ml IGF-I dose any better than 100 ng/ml suggests that either the concentration was still not high enough to saturate the IGFBP or that other mechanisms were responsible for a reduced IGF response. A possible IGFBP-independent mechanism is the inhibition of IGF receptor signaling by nitric oxide produced by OA chondrocytes[38]. The effects of aging and OAon the chondrocyte response to OP-1 have not been studied, but our results suggest that at least some response to OP-1 is maintained.

It should be noted that cell culture studies of chondrocyte IGF-I responsiveness are limited in their ability to completely predict the *in vivo* effects of therapeutic IGF-I. The response to IGF-I *in vivo* may be improved by the mechanical stimulation experienced by chondrocytes during joint loading and by the presence of other growth factors already present within the joint, as well as the matrix proteins present in articular cartilage. Indeed, some increase in proteoglycan production was noted in cartilage samples taken from some of the subjects in a recent trial of intraarticular IGF-I for OA (unpublished observations). Nevertheless, the results of our study would suggest that more research is needed to find ways to improve the response to IGF-I stimulation in older adults with arthritis in order to optimize its therapeutic use.

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