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Effects of insulin-like growth factor-1 and dexamethasone on cytokine-challenged cartilage: relevance to post-traumatic osteoarthritis

Y. Li † [a], Y. Wang † [a], S. Chubinskaya ‡, B. Schoeberl §, E. Florine §, P. Kopesky §, A.J. Grodzinsky † [*]

† *Massachusetts Institute of Technology, Cambridge, MA, USA*
‡ *Rush University Medical Center, Chicago, IL, USA*
§ *Merrimack Pharmaceuticals, USA*

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SUMMARY

Objective: Interleukin-1 is one of the inflammatory cytokines elevated after traumatic joint injury that plays a critical role in mediating cartilage tissue degradation, suppressing matrix biosynthesis, and inducing chondrocyte apoptosis, events associated with progression to post-traumatic osteoarthritis (PTOA). We studied the combined use of insulin-like growth factor-1 (IGF-1) and dexamethasone (Dex) to block these multiple degradative effects of cytokine challenge to articular cartilage.

Methods: Young bovine and adult human articular cartilage explants were treated with IL-1α in the presence or absence of IGF-1, Dex, or their combination. Loss of sulfated glycosaminoglycans (sGAG) and collagen were evaluated by the DMMB and hydroxyproline assays, respectively. Matrix biosynthesis was measured via radiolabel incorporation, chondrocyte gene expression by qRT-PCR, and cell viability by fluorescence staining.

Results: In young bovine cartilage, the combination of IGF-1 and Dex significantly inhibited the loss of sGAG and collagen, rescued the suppression of matrix biosynthesis, and inhibited the loss of chondrocyte viability caused by IL-1α treatment. In adult human cartilage, only IGF-1 rescued matrix biosynthesis and only Dex inhibited sGAG loss and improved cell viability. Thus, the combination of IGF-1 + Dex together showed combined beneficial effects in human cartilage.

Conclusions: Our findings suggest that the combination of IGF-1 and Dex has greater beneficial effects than either molecule alone in preventing cytokine-mediated cartilage degradation in adult human and young bovine cartilage. Our results support the use of such a combined approach as a potential treatment relevant to early cartilage degradative changes associated with joint injury.

Introduction

Post-traumatic osteoarthritis (PTOA) refers to the development of OA as a result of traumatic joint injury. PTOA accounts for 12% of the OA population and typically affects younger individuals[1,2]. The pathogenesis following acute joint injuries (such as anterior cruciate ligament rupture and/or meniscal tear) has been reported to involve an immediate increase in synovial fluid concentrations of pro-inflammatory cytokines (e.g., IL-1[3–5], TNF-α[3–7], and IL-6[3–7]), along with increased release of cartilage matrix fragments and noticeable chondrocyte apoptosis[8,9]. While cytokine levels eventually drop to that found in chronic OA[3], their continued presence upsets the balance between anabolic and catabolic processes in cartilage, which plays an important role in the progression to PTOA[10].

An ideal pharmacological intervention to prevent or delay the onset of PTOA should address several if not all of the pathogenic responses to joint injury; such intervention should inhibit inflammatory responses, prevent cell death, prevent cartilage matrix degradation, and promote production of new matrix[11]. Recent studies have explored the potential of several therapeutic approaches: IL-1 receptor antagonists (IL-1Ra) and TNF-α blockers to inhibit selected inflammatory mediators[10,12], metalloproteinase and aggrecanase inhibitors to reduce matrix degradation[10], and

* Address correspondence and reprint requests to: A. J. Grodzinsky, MIT NE47-377, Cambridge, MA 02139, USA. Tel: 1-617-253-4969; Fax: 1-617-258-5239.
E-mail address: alg@mit.edu (A.J. Grodzinsky).
[a] Contributed equally to this manuscript.

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growth factors such as BMP-7 and FGF-18 that promote cartilage matrix production and cell proliferation and counteract matrix degradation[13]. However, these studies all focused on targeting a single, specific pathway/molecule; many investigators now believe that no single compound will be sufficient to treat every aspect of OA[14], and that a combination of therapeutics will be necessary to address multiple pathogenic mechanisms.

In the current study, we take a novel approach by systematically studying the potential of a combination therapeutic to achieve both pro-anabolic and anti-catabolic effects, and at the same time, to improve cell viability. Insulin-like growth factor-1 (IGF-1) is a potent growth factor that can upregulate cartilage matrix biosynthesis and, under certain circumstances, inhibit catabolic processes stimulated by cytokines[15,16]. IGF-1 binds to the chondrocyte IGF-1 receptor and transduces signals *via* the IRS-1/PI3K/Akt pathway, which regulates protein synthesis[17]. In addition, IGF-1 provides pro-survival signals and has been shown *in vitro* to rescue chondrocyte apoptosis induced by collagen degradation[18] and by mechanical injury[19]. Dexamethasone (Dex), a potent synthetic glucocorticoid (GC), has been widely used intra-articularly to relieve inflammation for the treatment of OA and other arthritis[20]. Dex has been shown to block cytokine-induced cartilage matrix catabolism and to alleviate cytokine-induced inhibition of matrix biosynthesis in bovine cartilage *via* GC receptor-dependent pathways[21]. The ability of this combination of IGF-1 and Dex to modulate cytokine-mediated cartilage degradation and to simultaneously maintain chondrocyte viability in the face of cytokine challenge remains to be elucidated.

Using adult human knee and ankle cartilage and young bovine cartilage in an *in vitro* model system, our objectives were to quantify the effects of IGF-1, Dex and their combination on IL-1-induced degradation of aggrecan and collagen, inhibition of proteoglycan biosynthesis, and altered chondrocyte viability. Furthermore, we examined the hypothesis that the effects of IGF-1 and Dex are consequences of their direct transcriptional regulation by comparing changes at the protein level to their effects at the level of gene transcription.

Materials and methods

Bovine cartilage harvest and culture

Cartilage disks were harvested from the femoropatellar grooves of 1–2-week-old calves (obtained from Research 87, Boylston, MA). Explants were harvested within 8 h after animal death and a total of 15 joints from 14 animals were used. Briefly, a 3-mm dermal punch was used to core full-thickness cartilage cylinders, and the top 1-mm disk containing intact superficial zone was obtained with a blade. For each experiment, disks from different treatment groups were matched for anatomic location along the joint surface. Explant disks were then equilibrated in serum-free medium: low glucose Dulbecco's Modified Eagle Medium (DMEM); 1 g/L (Corning Cellgro, Manassas, VA) supplemented with 10 mM HEPES buffer (Gibco, Grand Island, NY), 0.1 mM nonessential amino acids (Sigma Aldrich, St. Louis, MO), 0.4 mM proline (Sigma), 20 μg/ml ascorbic acid (Sigma), 100 units/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Sigma) for 2–3 days (5% CO_2; 37°C). Serum-free conditions were chosen to distinguish the specific effects of exogenous IGF-1 from the unknown concentrations of endogenous growth factors that may be present in serum.

Adult human cartilage harvest and culture

Cartilage from adult human knee and ankle joints was obtained postmortem from the Gift of Hope Organ and Tissue Donor Network (Itasca, IL). All procedures were approved by the Rush University Medical Center Institutional Review Board (ORA Number: 08082803-IRB01-AM01) and the Committee on the Use of Humans as Experimental Subjects at MIT. At the time of donor tissue harvest, the joint surfaces were scored by an experienced forensic pathologist using the modified Collins grading system[22]. 20 joints from 13 donors were used in this study (an ankle/knee pair was obtained from one donor, and ankle pairs were obtained from six donors). 14 ankle joints (Collin's grade 1) were from eight donors, age 64–76 years old (see Supplementary Table S1 for enumeration of joints). Six knee joints (Collin's grade 0–2) were from six donors, age 19–66 years old. Full-thickness (~1–2 mm) cartilage disks cored with a 3-mm punch were harvested from the talar domes of ankles and the tibial plateau or distal femur of knees. Explants were harvested within 24–36 h after death of donors and only unfibrillated cartilage was used. Joint surfaces excluded from the study were the visibly roughened surfaces where the superficial layer had clearly lost its smooth architecture and the collagen appeared to be fibrillated. In order to be consistent with a previous study using human tissue[23], human explants were equilibrated for 2–3 days in high glucose DMEM (4.5 g/L; Corning Cellgro, Manassas, VA) containing the same supplements as in the bovine culture medium. To determine the effects of glucose concentration on cartilage response, a separate control study was performed using explants from three additional knee joints and six additional ankle joints (Table S1, Fig. S5) cultured in low glucose DMEM with otherwise identical supplements.

Dose responses of IGF-1 and Dex in the presence of IL-1

Many studies of cartilage explants treated with IL-1 have been reported utilizing IL-1 concentrations ranging from 0.05 to 100 ng/ml[15,24]. Based on our own preliminary dose–response study of sGAG loss vs IL-1α (R&D Systems, Minneapolis, MN) at 1, 2, 5, and 10 ng/ml) with bovine cartilage, we chose an IL-1α concentration of 1 ng/ml for all the present experiments, representing a moderately aggressive cytokine treatment. We then tested the effects of Dex (Sigma) alone (at 10 nM, 100 nM and 1 μM) and IGF-1 (R&D Systems) alone (at 10, 100 and 300 ng/ml) on sGAG loss and proteoglycan biosynthesis in bovine cartilage treated with 1 ng/ml IL-1α. Based on the results of these dose response studies [Fig. 1(A and B)], we chose concentrations of 100 nM Dex and 100 ng/ml IGF-1, both alone and in combination, for all subsequent tests of sGAG loss, biosynthesis, gene expression and cell viability.

Biosynthesis, sGAG, and biochemical analysis

Cartilage disks were radiolabeled with 5 μCi/ml ^{35}S-sulfate (Perkin-Elmer, Norwalk, CT) for 36–48 h. When terminated, disks were washed in PBS, weighed and digested with proteinase K (Roche, Indianapolis, MN). Radiolabel incorporation was measured using a liquid scintillation counter (PerkinElmer), and normalized to DNA for bovine explants (measured *via* Hoechst 33258 dye-binding assay[25]), and to wet weight for human explants. The DMMB dye-binding assay[26] and hydroxyproline assay[27] was used to determine the sGAG content and collagen content, respectively, of digested cartilage explants and medium. The sGAG and collagen content data were expressed as a percentage of the total sGAG or collagen, respectively.

Cell viability

Slices (100–200 μm thick) were cut with a scalpel from the center of cartilage disks[28] (see Supplementary Figure S1 for slice orientation) and incubated for 2–3 min in the dark in PBS containing fluorescein diacetate (FDA; 4 μg/ml) and propidium iodide (PI; 40 μg/ml) (both from Sigma), for viable and non-viable cell

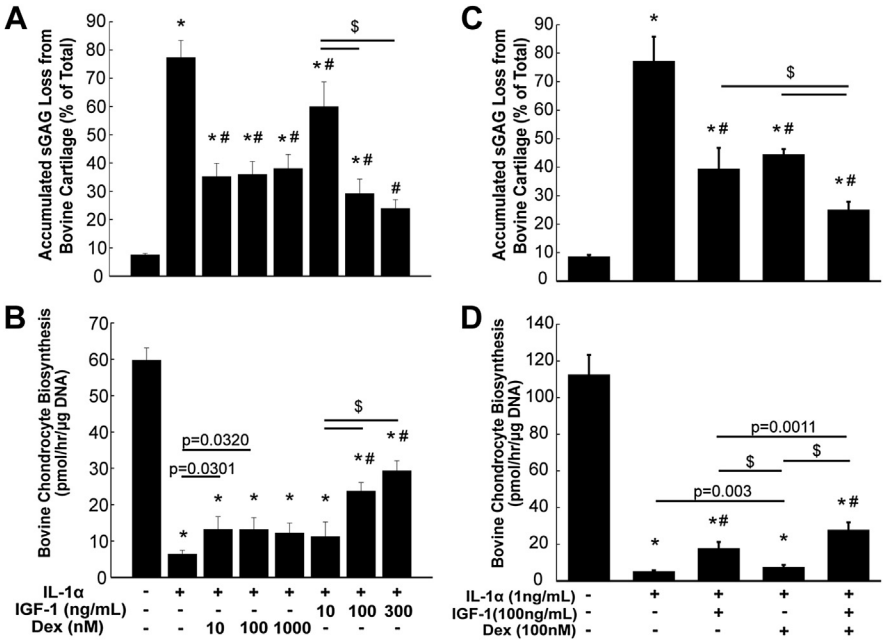

Fig. 1. **A**, Percent sGAG loss from immature bovine cartilage in an 8-day dose response experiment. Disks were subjected to different doses of Dex or IGF-1 in the presence of IL-1α (1 ng/ml); 23–24 disks/condition from $n = 4$ animals. **B**, Normalized sulfate incorporation rate measured during Day 6–8 of the same disks in A. **C**, Percent sGAG loss in response to 8-day treatments using 18 disks/condition from $n = 3$ animals. **D**, Normalized sulfate incorporation rate measured during Day 6–8 of the same disks used in **C**. Values are mean and 95% confidence interval, * vs untreated control ($P < 0.0001$); # vs IL-1α alone ($P < 0.0001$); $: $P < 0.0001$.

staining, respectively. After two washes with PBS, the entire 1 mm × 3 mm area of each slice from superficial surface to 1 mm deep was imaged using a Nikon fluorescence microscope at 4× magnification. 1–2 slices were imaged (2 images/slice) for each sample. For human cartilage disks, cell viability was quantified using ImageJ. The numbers of viable and non-viable cells were counted over each entire slice *via* the Image-based tool for Counting Nuclei (ITCN version 1.6) plug-in, and data were expressed as percent of total viable cells.

Gene expression analyses

Bovine cartilage disks from five animals (six disks per condition per animal) were treated for 4 days and stored in −80°C after flash-freezing. Using methods described in detail previously[29,30], the six disks from each condition were pooled, pulverized, and homogenized in TRIzol reagent, and then separated using phase-gel tubes (Eppendorf, Hamburg, Germany). The supernatant was purified following the Qiagen RNeasy mini kit protocol (Qiagen, Chatsworth, CA). Equal amounts of mRNA from each condition were reverse transcribed using the AmpliTaq-Gold Reverse Transcription kit (Applied Biosystems, Foster City, CA). Primer pairs used were previously reported[29,30] except for caspase-3: forward 5′-GAAGTCTGACTGGAAAACCC-3′, reverse 5′- GAAGTCTGCCTCAACTGGTA-3′. Real-time PCR were performed using the Applied Biosystems 7700HT instrument with SYBR Green Master Mix (Applied Biosystems). Expression data for each gene were calculated from the threshold cycle (Ct) value, and normalized to the internal housekeeping gene 18S. Our previous studies explored several housekeeping genes and normalization methods and, in the system used here, in the absence of impact mechanical injury to cartilage, normalization to 18s and G3PDH gave similar results[29,30].

Statistical analysis

Bovine and human sGAG loss and biosynthesis data, bovine collagen loss data, bovine gene expression data, and human viability data were analyzed using the linear mixed effects model with animal (or human donor) as a random factor, followed by Tukey's Honestly Significant Difference (Tukey's HSD) test for pairwise comparisons. Statistical tests were performed using either Systat 12 or JMP Pro 11 software. *P*-values less than 0.05 were considered statistically significant.

Results

Combined Dex plus IGF-1 treatment is most effective in ameliorating IL-1α-induced GAG loss and inhibition of biosynthesis in bovine cartilage

The effects of IGF-1, Dex, and the combination of IGF-1 and Dex on IL-1α-treated bovine cartilage were examined in three independent 8-day experiments (three animals). Consistent with previous reports, IGF-1 alone and Dex alone were each able to reduce the loss of sGAG caused by IL-1α treatment [Fig. 1(C)]. Similarly, IGF-1 alone and Dex alone partly reversed the inhibition of proteoglycan biosynthesis caused by IL-1α [Fig. 1(D)], though Dex was less effective than IGF-1. However, the combination of IGF-1 + Dex showed significant further reduction in sGAG loss and higher biosynthesis compared to either Dex or IGF-1 alone. The concentration of Dex and IGF-1 used for the combination study was based on these dose response experiments [Fig. 1(A and B), and Supplementary Fig. S2 which shows separately the sGAG content remaining in explants and that lost to the medium by Day 8 corresponding to the experiments of Fig. 1(A)].

The combined effects of IGF-1 and Dex on collagen degradation

Bovine explants were cultured in IL-1α medium for 25 days to study the kinetics of sGAG and collagen release to the medium. During the first 10 days, IL-1α induced significantly more sGAG loss compared to untreated controls, consistent with the previously reported activity of ADAMTS-4[31] [Fig. 2(A), $P < 0.0001$], while collagen release was negligible from both control and IL-1α-treated cartilage. By Day 12, when ~85% sGAG was depleted from IL-1α-

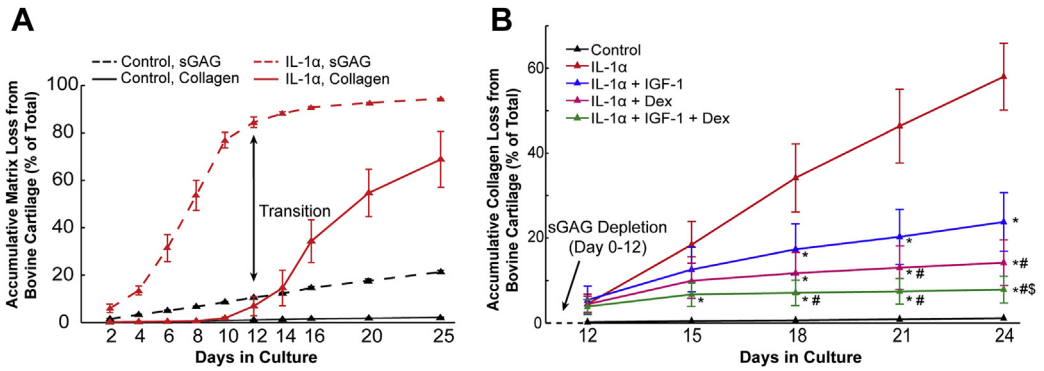

Fig. 2. **A**, Kinetics of sGAG and collagen (OH-proline) loss from immature bovine cartilage disks in response to 25-day treatment with 1 ng/ml IL-1α; $N = 6$ disks. **B**, Accumulated collagen loss to the medium from Day 12–24 for cartilage disks in response to 24-day treatments. IL-1α (1 ng/ml) was added on Day 0 while IGF-1 (100 ng/ml) and Dex (100 nM) were introduced on Day 12 after the majority of sGAG was depleted; 18–20 disks/condition from $n = 3$ independent experiments. Values are mean and 95% confidence interval, * vs IL-1α alone; # vs IL-1α + IGF-1; $ vs IL-1α + Dex, $P < 0.05$.

treated disks, collagen release became greater than controls (6.9% vs 1.1%, $P = 0.0378$). By Day 25, collagen loss reached 68.8% for IL-1α treatment vs 2.1% for controls [Fig. 2(A); $P < 0.0001$].

Next, we examined the effects of IGF-1 and Dex on the extent of IL-1α-induced collagen release that would occur after substantial sGAG had already been lost, as a model for treatment during the earliest stages of PTOA-like inflammatory challenge (three independent experiments with three animals). IL-1α treatment was maintained over the entire 24-day study, but IGF-1, Dex, or the combination was not introduced until Day 12, by which time ~70% GAG release had already occurred. (Total sGAG content remaining in the explants after 24 days of culture and that lost to the medium from day 0–24 are shown separately in Supplementary Fig. S3.)

During days 12–24, IL-1α alone caused cumulative collagen release of 58% vs 1.1% in the untreated controls [Fig. 2(B); $P < 0.0001$]. This collagen release was markedly attenuated by IGF-1 alone to 23.8% ($P < 0.0001$) and by Dex alone to 14.2% ($P = 0.008$ vs IL-1α + IGF-1). Combination treatment with IGF-1 and Dex reduced collagen loss to only 7.9% ($P = 0.028$ vs IL-1α + Dex). Collagen release kinetics revealed that the effects of IGF-1 ($P = 0.0113$) or Dex ($P < 0.0001$) alone, became significantly different from IL-1α by Day 18 [Fig. 2(B)], while combined [IGF-1 + Dex] ($P = 0.001$) treatment became significant by Day 15. Furthermore, the combination treatment completely arrested collagen release between Days 15–24 [Fig. 2(B)]. (Total hydroxyproline content remaining in explants after 24 days of culture and that lost to the medium from days 12–24 are shown in Supplementary Fig. S4.)

IGF-1 and Dex maintained bovine chondrocyte viability in the presence of IL-1α

Bovine disks were cultured in IL-1α medium with or without Dex for 8, 16, and 24 days [Fig. 3(A)]. Increased cell death was observed as early as 8 days after IL-1α treatment, and by Day 24, disks had undergone severe degeneration [Fig. 3(A), bottom-middle column]. In contrast, addition of Dex greatly reduced IL-1α-induced cell death and its effects were sustained over 24 days. In separate experiments [Fig. 3(B)], IGF-1, Dex, or both greatly reduced

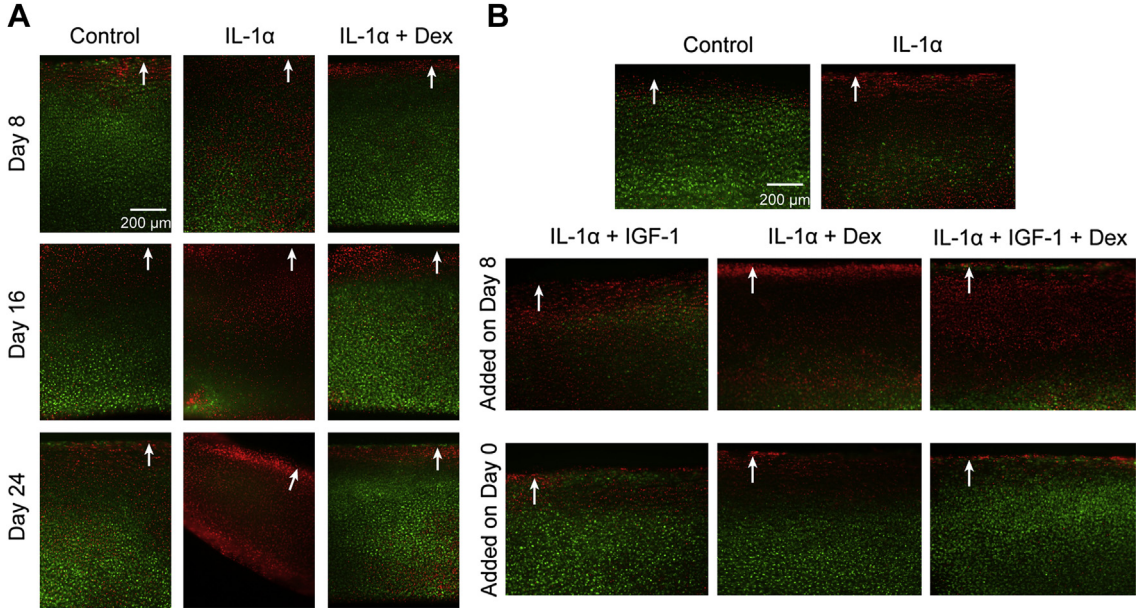

Fig. 3. **A**, Bovine chondrocyte viability in cartilage disks in response to 8, 16, or 24-day treatments. Treatment groups are control, IL-1α (1 ng/mL), and IL-1α + Dex (100 nM). Cells were fluorescently labeled with fluorescein diacetate (green, viable) and propidium iodide (red, non-viable). **B**, Bovine chondrocyte viability evaluated on Day 16 after treatments. Disks were treated with IL-1α (1 ng/ml) for the first 8 days, and switched to 1 pg/ml IL-1α between Day 8 and Day 16. IGF-1 (100 ng/ml), Dex (100 nM), or both were added either on Day 8 (middle panel) or Day 0 (bottom panel). White arrow: intact superficial surface of cartilage. Scale bar = 200 μm.

IL-1α-induced cell death when introduced on Day 0, but such rescuing effects were not observed when added on Day 8.

Bovine chondrocyte gene expression

mRNA levels of aggrecan and collagen II were significantly downregulated by IL-1α after 4-day treatment (Fig. 4). The addition of Dex or the combination reversed downregulation of aggrecan expression compared to IL-1α alone; the effect of Dex was greater than that of IGF-1. In contrast, only the combination treatment reversed inhibition of collagen II expression (Fig. 4). ADAMTS-4 expression was upregulated by IL-1α, but was suppressed by IGF-1, Dex, or the combination. IL-1α increased ADAMTS-5 expression by greater than 200-fold, which was reversed by IGF-1 or Dex alone; the combination showed a further reduction compared to either treatment alone. IL-1α also elevated mRNA levels of the proprotein convertases PACE-4 and Furin, which in turn were significantly suppressed by the combination of IGF-1 and Dex (Fig. 4). MMP-3 and MMP-13 were similarly upregulated several hundred fold by IL-1α; adding IGF-1 had no effect on either gene ($P = 0.103$ for MMP-3; $P = 0.057$ for MMP-13), whereas Dex or the combination markedly reduced both genes (Fig. 4). mRNA levels of COX-2 were upregulated by IL-1α, but suppressed by IGF-1, and further reduced by Dex (Fig. 4). IL-1α significantly increased iNOS expression, but IGF-1, Dex, or their combination had no additional effect. Dex completely blocked IL-1α-induced increases in IL-6 expression, while IGF-1 showed no effect at all. The apoptosis executioner gene caspase-3 was increased by IL-1α treatment, but was significantly reduced by Dex or Dex + IGF-1; however, IGF-1 by itself had no suppressive effect (Fig. 4).

Only Dex suppressed IL-1-induced GAG loss in human cartilage

sGAG loss from human ankle and knee cartilage induced by IL-1α was evaluated in a 17-day study (eight independent experiments, five for ankle and three for knee). IL-1α significantly increased sGAG loss compared to untreated controls [Fig. 5(A and C); $P < 0.0001$ for both ankle and knee cartilage]. Dex completely blocked sGAG loss compared to IL-1α alone ($P < 0.0001$, both ankle and knee) to a level not different from the untreated controls ($P = 0.0762$ for ankle; $P = 0.5418$ for knee). However, the addition of IGF-1 did not reduce elevated sGAG release. The combination of Dex and IGF-1 showed similar results as Dex alone. As these human explant cultures were maintained in high glucose DMEM and the bovine cultures above were in low glucose DMEM, we conducted additional experiments with six ankle joints (three donors) and three knee joints (three donors, Table S1) to test whether glucose concentration altered the catabolic response of these human tissues. We found that glucose concentration does not change our conclusion regarding the ability of Dex to suppress sGAG loss in adult human donor tissues under cytokine challenge, whether by IL-1 or [TNFα + IL-6/sIL-6R] (Supplementary Fig. S5).

Only IGF-1 could rescue chondrocyte biosynthesis in human cartilage

In the same 17-day study with human cartilage explants (Fig. 5), ^{35}S-sulfate incorporation rates as a measure of proteoglycan biosynthesis were measured during Day 15–17. Compared to untreated controls, biosynthesis was suppressed by IL-1α treatment in

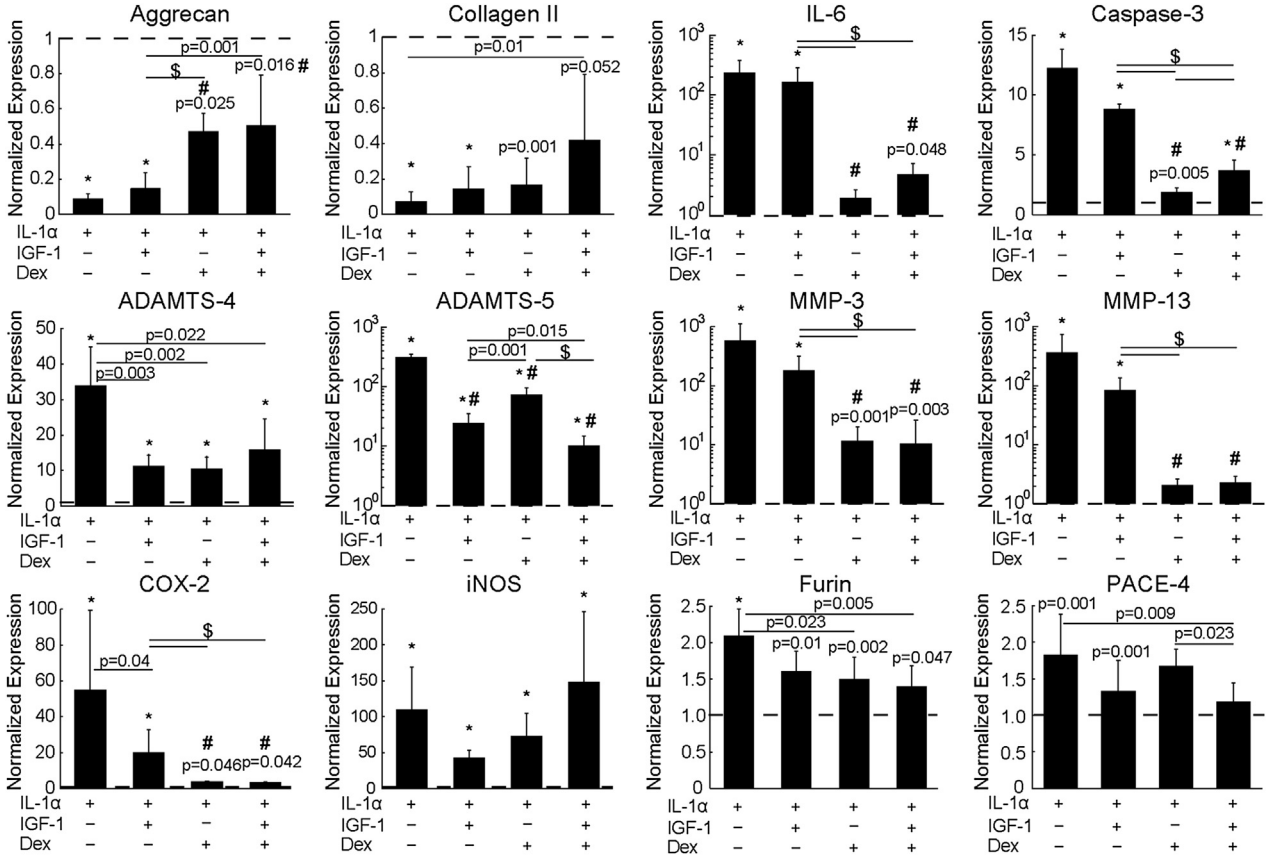

Fig. 4. The effects of IGF-1 (100 ng/ml) and Dex (100 nM) on bovine chondrocyte gene expression after 4-day treatment with IL-1α (1 ng/ml). For each condition, six cartilage disks from the same animal were pooled for mRNA extraction; $n = 5$ animals. Gene expression levels were normalized to that of the 18S gene and then normalized to the untreated control condition which had an expression level = 1 (dotted line). Data are presented as mean ± 95% confidence interval, * vs untreated control ($P < 0.001$); # vs IL-1α alone ($P < 0.001$); $:$P < 0.001$.

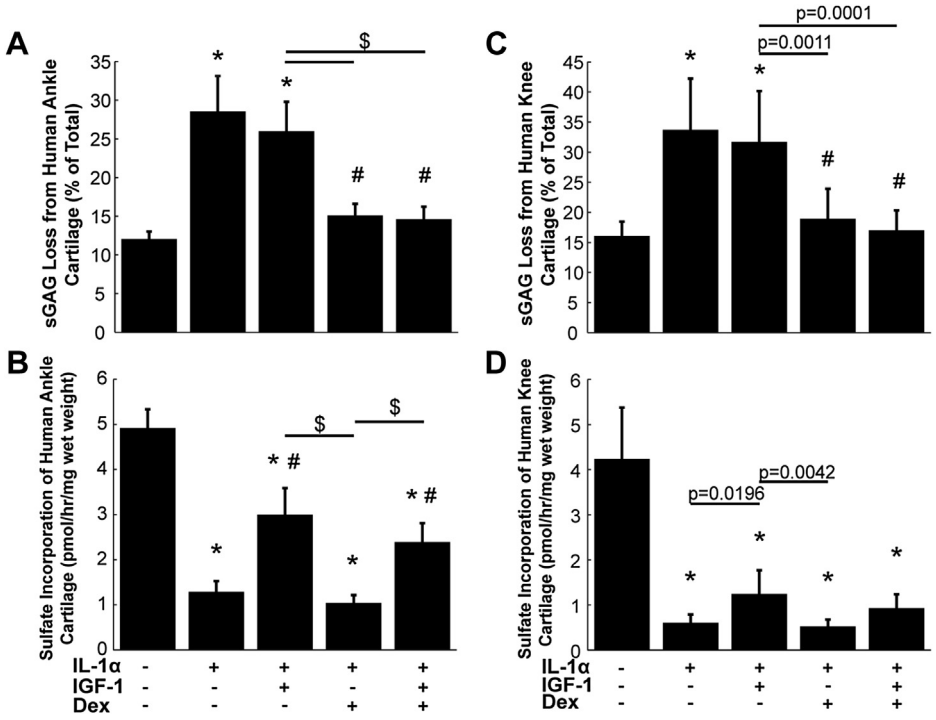

Fig. 5. **A**, Percent sGAG loss from adult human ankle cartilage in response to 17-day treatments, $N = 40$ disks from $n = 5$ donors (one independent experiment per donor). **B**, Normalized sulfate incorporation rate during Day 15–17 for the same cartilage disks used in **A** above. **C**, Percent sGAG loss from adult human knee cartilage in response to 17-day treatments, $N = 24$ disks from $n = 3$ donors (1 independent experiment per donor). **D**, Normalized sulfate incorporation rate during Day 15–17 of the same disks in **C**, Values are mean and 95% confidence interval. * vs untreated control ($P < 0.0001$); # vs IL-1α alone ($P < 0.0001$); $: $P < 0.0001$.

both ankle and knee cartilage [Fig. 5(B and D); $P < 0.0001$ for both]. IGF-1 reversed this inhibition of biosynthesis in the ankle ($P < 0.0001$) and knee ($P = 0.0196$), whereas Dex had no effect ($P = 0.3452$ in ankle, $P = 0.979$ in knee). The combination of IGF-1 and Dex significantly increased biosynthesis but the effect was similar to IGF-1 alone ($P = 0.3936$) in the ankle. IGF-1 + Dex did not reverse the effects of IL-1α treatment in knee cartilage ($P = 0.3194$), but was similar to the effect of IGF-1 alone ($P = 0.7764$).

Only Dex maintained human chondrocyte viability in the presence of IL-1α

Chondrocyte viability in human cartilage explants was evaluated 17 days after treatment on two ankle joints and a knee joint. Representative images indicated significant cell death occurred under IL-1α treatment [Fig. 6(A)], especially in the superficial zone, and only Dex treatment rescued cell death. Quantitative results

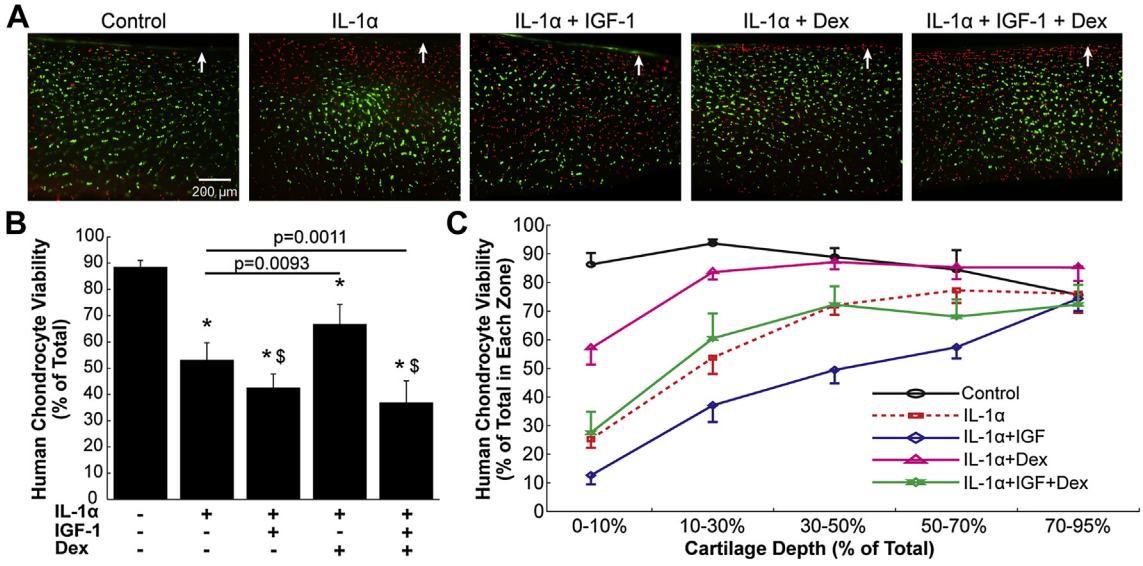

Fig. 6. **A**, Representative images of fluorescently stained adult human ankle cartilage on Day 17 after treatments. Cells were labeled with fluorescein diacetate (green, viable) and propidium iodide (red, non-viable). White arrow: superficial surface. Scale bar = 200 μm. **B**, Quantified percent viability from red/green images of two adult human ankles and one human knee cartilage (64-yr-old female ankle, $N = 6$ disks; 66-yr-old female ankle, $N = 4$ disks; same 66-yr-old female knee, $N = 12$ disks). Data are presented as mean ± 95% confidence interval, * vs untreated control; $ vs IL-1α + Dex, P value <0.0001. **C**, Depth-dependent cell viability in the 64-yr-old female ankle cartilage. Each slice of a disk was divided into five zones, with 0–10% as the superficial zone. The deepest 5% depth was not included in the analysis due to cutting-induced cell death.

[Fig. 6(B)] revealed similar trends for both ankle and knee cartilage; specifically, IL-1α induced significant cell death compared to the untreated controls [Fig. 6(B)]. Addition of IGF-1 showed no rescue of cell viability while addition of Dex rescued overall viability of chondrocytes in the presence of IL-1α. The combination of IGF-1 and Dex showed no significant difference compared to IGF-1 alone ($P = 0.6789$) in rescuing cell viability. A majority of IL-1α-induced cell death occurred in the superficial and middle zones of ankle cartilage [Fig. 6(C)], and only the addition of Dex rescued cell death throughout the entire depth of the cartilage disks.

Discussion

We tested the hypothesis that the combination of potent anti-catabolic and pro-anabolic agents could ameliorate degradative effects of inflammatory cytokines known to be upregulated during the earliest stages following acute joint injury. Using IL-1α as a model of cytokine challenge *in vitro*, our results demonstrated that IGF-1 and Dex complemented each other in adult human cartilage; specifically, IGF-1 promoted matrix biosynthesis while Dex blocked matrix loss and preserved cell viability. Importantly, these beneficial effects could not be achieved by either agent alone. In young bovine cartilage, both IGF-1 and Dex provided pro-anabolic and anti-catabolic effects, and these combined effects were additive or synergistic. While Dex preserved cell viability during IL-1α challenge in both adult human and young bovine cartilage, IGF-1 only offered protection in young bovine cartilage. Lastly, we hypothesize that the observed changes at the protein level are consistent with the observed transcriptional upregulation by IGF-1 and Dex of aggrecan and collagen II gene expression, and suppression on MMPs, aggrecanases ADAMTS-4 and -5 mRNA, as well as proprotein convertases Furin and PACE 4.

We found for the first time that for the case of human articular cartilage, Dex was more effective in maintaining chondrocyte viability and minimizing GAG loss, while IGF-1 was more effective at reversing the effects of cytokine-induced inhibition of GAG synthesis; thus, the use of a combined therapeutic approach was better than either individual treatment alone. The combined ability of Dex and IGF-1 treatment to protect against collagen degradation even after substantial aggrecan GAG loss has already occurred [Fig. 2(B)] is demonstrated clearly using bovine articular cartilage. Together, these results suggest that it may be possible to preserve the matrix fibrillar structure and enable time for replenishment of aggrecan (and other matrix constituents) given suitable pro-anabolic stimulation of biosynthesis by viable chondrocytes, wherein cell viability and broad spectrum inhibition of cytokine-induced degradation can be maintained by simultaneously delivered factors.

Certain mechanisms by which [IGF-1 + Dex] can prevent proteoglycan degradation in the presence of IL-1α appear to manifest at the level of gene transcription. In addition to inhibition of aggrecanase transcription (Fig. 4), IGF-1 has been reported to reduce the abundance of MMP-13 protein secreted by chondrocytes and to decrease mRNA levels of certain MMPs[32], though the decrease in MMP-13 expression in Fig. 4 was not significant. Here, we have added new information by showing that IGF-1 can suppress collagen loss even after the majority of sGAG is depleted by IL-1α [Fig. 2(B)]. We found that Dex may further protect collagen by suppressing MMP-13 expression (Fig. 4), consistent with previous studies[33,34]. Thus, the combination of Dex and IGF-1 provides marked protection of collagen that cannot be achieved by either agent alone.

The strong increase in IL-6 mRNA levels by IL-1α suggests that the effects of IL-1α may be augmented by IL-6. Interestingly, Dex strongly suppressed IL-6 gene expression (Fig. 4). Guerne *et al.*[35] showed that Dex (100 nM) rescued the inhibition of PG synthesis caused by IL-6/sIL-6 or IL-6 observed in primary human chondrocyte culture. Dex likely counteracts the suppressive effects of inflammatory cytokines on matrix biosynthesis rather than directly promoting biosynthesis.

A surprising finding of this study is that Dex prevented IL-1α-induced chondrocyte cell death in both young bovine and adult human cartilage. We know of no previous report describing the effects of Dex on cytokine-induced chondrocyte cell death in human cartilage explants. In the literature, the effects of Dex on chondrocyte death have been contradictory. Comparison between studies is difficult because of the widely varying Dex dosages used, differences in cell types, culture conditions, the use of isolated cells vs explants *in vitro*, differences in animal species *in vivo*, and differences in outcomes measures used. For example, Dex induced apoptosis in proliferative chondrocytes cell lines[36,37], isolated primary chondrocytes[38], and in terminally differentiated hypertrophic chondrocytes[39]. In contrast, Dex prevented apoptosis in terminally differentiated murine cell lines[40] and did not compromise cell viability in long-term culture of bovine cartilage explants[41]. These findings suggest that the pro- or anti-apoptotic nature of Dex is cell type-, cell differentiation stage-, dose-, as well as stimulus-dependent. Therefore, when cartilage is challenged with IL-1α, we speculate that Dex interferes with cytokine-induced apoptotic signaling network. This is supported by the downregulation of caspase-3 mRNA expression by Dex in the present study. The mechanistic pathways involving transduction of the effects of Dex on musculoskeletal cells have been widely discussed in the literature[42–45]. Our ongoing studies utilize a phosphoproteomics approach focused on pathways by which Dex may regulate chondrocyte apoptosis, though this is beyond the scope of the present study.

In vivo, both chondroprotective and deleterious effects have both been reported upon intra-articular injection of GCs. Intra-articular injection of GCs typically involves very high doses (e.g., up to ~100 mM[44,46], compared to the 100 nM Dex concentrations used in the present study). Such high-dose intra-articular injections[47,48] have caused systemic side effects. Thus, therapeutic efficacy would be greatly aided by methods to deliver low concentrations of Dex directly inside cartilage so as to appropriately minimize exposure of Dex to other joint tissues such as tendon, ligament and meniscus[28].

In contrast to our findings with young bovine cartilage, both the anti-catabolic and pro-cell-survival effects of IGF-1 were lost with adult human ankle and knee cartilage. Substantial evidence in the literature has shown that OA and aging chondrocytes respond poorly to IGF-1[49,50], due to the presence of IGF-1 binding proteins (IGF-BPs) in the matrix[51,52] and to altered intracellular signaling by mediators such as reactive oxygen species[53]. Yin *et al.*[17] demonstrated that oxidative stress is responsible for inhibiting Akt phosphorylation and stimulating MEK-ERK MAPK signaling in human OA chondrocytes and, as a result, IGF-1 signaling was blocked along with proteoglycan synthesis. In the present study, we found that IGF-1 could significantly rescue IL-1α-suppressed proteoglycan biosynthesis in human cartilage but did not affect proteoglycan degradation. Ongoing studies of these differences in the response between human and bovine cartilage to such combination therapeutics provide a unique opportunity to determine the mechanisms which account for cell sensitivity to IGF-1 or Dex treatment. An understanding of these mechanisms will aid in translating these *in vitro* data to clinical applications.

In summary, we demonstrated the beneficial effects of the combination of IGF-1 and Dex in reversing IL-1α-suppressed biosynthesis and blocking cytokine-induced proteoglycan and collagen degradation in young bovine explants. Furthermore, IGF-

1 + Dex strongly inhibited cell death induced in an inflammatory environment. Importantly, we showed that each therapeutic factor has a unique role in cytokine-challenged adult human cartilage: IGF-1 stimulated proteoglycan biosynthesis while Dex ameliorated cartilage catabolism. Consequently, the result from this study highlights the need to investigate combination therapeutics in clinical settings. The advantageous effects of this combination therapy could be further enhanced by using a cartilage matrix binding variant of IGF-1[54] as well as appropriate *in vivo* delivery methods, e.g., intra-articular injection with cartilage-homing nanoparticles that may be relevant for treatment of early stage PTOA[28].

Contributions

YL and YW contributed to the conception and design of the study, including collection, analysis, and interpretation of data, drafting and revising of the manuscript. PK and BS contributed to the conception and design of the study. SC contributed to obtaining and interpretation of data on human tissue. EF and PK conducted the experiments shown in Fig. S5 and critically reviewed the revised manuscript. AJG contributed to the conception and design of the study, including interpretation of data and drafting and critical revision of the article for intellectual content. All authors approved the final version of the article. Responsible authors for integrity of the study: YL YW AJG.

Conflict of interest

All authors have no financial interests or personal relationships with other people or organizations that could potentially and inappropriately influence the conduct or reporting of this work.

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Supplementary data

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