Metabolic and mitogenic activities of insulin-like growth factor-1 in interleukin-1-conditioned equine cartilage

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Objective—To determine response of interleukin-1α (IL-1α)-conditioned equine articular cartilage explants to insulin-like growth factor-1 (IGF-1).

Sample Population—Cartilage from the trochlea and condyles of the femur of a clinically normal 4-year-old horse.

Procedure—Effects of IGF-1 (0 to 500 ng/ml) after addition of IL-1α were evaluated by assessing matrix responses, using a sulfated glycosaminoglycan (GAG) assay, matrix 35SO4 GAG incorporation, and release of GAG. Mitogenic response was assessed by 1H-thymidine incorporation into DNA and fluorometric assay of total DNA concentration.

Results—Human recombinant IL-1α (40 ng/ml) increased the amount of labeled GAG released and decreased labeled and total GAG remaining in explants, and IL-1α decreased mitogenic response. Addition of IGF-1 counteracted effects seen with IL-1α alone. In general, IGF-1 decreased total and labeled GAG released into the medium, compared with IL-1α–treated explants (positive-control sample). Values for these variables did not differ significantly from those for negative-control explants. A significant increase in total and newly synthesized GAG in the explants at termination of the experiment was observed with 500 ng of IGF-1/ml. Labeled GAG remaining in explants was greater with treatment at 50 ng of IGF-1/ml, compared with treatment with IL-1α alone. Concentrations of 200 ng of IGF-1/ml abolished actions of IL-1α and restored DNA synthesis to values similar to those of negative-control explants.

Conclusions and Clinical Relevance—IGF-1 at 500 ng/ml was best at overcoming detrimental effects associated with IL-1α in vitro explants. These beneficial effects may be useful in horses with osteoarthritis. (Am J Vet Res 2000;61:436–441)
Therefore, a combination treatment (such as corticosteroids) that suppresses mediators of OA without suppressing chondrocytic matrix synthesis, or that even enhances synthesis (such as IGF), would be desirable. This approach has had preliminary success in other species, using in vitro models.30,37

A study was conducted on IL-1α-conditioned equine explants from normal horses, using a single-dose regimen of a combination of corticosteroids and IGF-1. The doses used in that study were chosen empirically on the basis of a review of the literature. Analysis of results indicated that normal cartilage cultured after addition of the combination treatment had a significant increase in the amount of total GAG and labeled GAG that was retained in the cartilage, compared with control values. The combination actually was associated with a significant increase in the measured variables, compared with values for corticosteroid or IGF-1 treatments alone. Unfortunately, the effect of the combination treatment was not as substantial in explants incubated with IL-1α. The purpose of the study reported here was to conduct a dose titration of IGF-1 in equine articular cartilage explants incubated with IL-1α. The objective was to identify an optimal dosage of IGF-1 that could be used in a combination treatment in the presence of IL-1α.

Materials and Methods

Collection of cartilage—Cartilage was collected from the articulating surfaces of the trochlea and condyles of the distal aspect of the femur from a clinically normal 4-year-old castrated male horse. Care was taken to ensure calcified cartilage was not harvested. At the time of collection, examination of the cartilage did not reveal grossly visible abnormalities. Collection and handling of cartilage was performed in an aseptic manner, and cartilage samples were bathed in a Gey balanced salt solution.7

Culture of cartilage—Cartilage explants were cut to a size of approximately 25 mm², which corresponded to a wet weight of 60 to 100 mg. One cartilage explant was cultured per well in 24-well plates incubated at 37°C and 5% CO2. Explants were covered by high-glucose Dulbecco’s modified Eagle’s medium containing 10% equine serum and supplemented media was used in the negative-control group (0 ng of IGF-1/ml) and groups at various doses of IGF-1 (10, 50, 200, and 500 ng of IGF-1/ml, respectively). Media were collected and changed every 2 days, new treatments were applied at similar intervals, and all explants were cultured for 8 days after application of the first treatment. All samples (media and cartilage) were frozen at −70°C at the time of collection, unless otherwise indicated.

Sixteen hours prior to the beginning of the first experiment (pulse-chase labeling before treatment), 20 µCi of 35SO4/ml was added to each well. At the end of 16 hours, each explant was rinsed 3 times in fresh media followed by the addition of the aforementioned doses of IGF-1. Sixteen hours prior to termination of the second experiment (pulse-chase labeling after treatment), 10 µCi of 35SO4/ml and 5 µCi of H-thymidine/ml were added to each well. After termination of both experiments, explants were rinsed in a Gey balanced salt solution and frozen at −70°C for subsequent analysis.

Before analysis, all cartilage explants were lyophilized for 24 hours to remove all H2O and weighed. One milliliter of 2X papain7 (0.5 mg/ml) was added per 10-mg quantity of lyophilized cartilage explant, and the mixture was heated at 65°C for 24 hours to solubilize cartilage GAG. A similar method of ensuring solubilization of GAG in the media was performed, using papain 1:1 (vol:vol), but samples were heated for only 4 hours.

Total GAG quantification—The amount of GAG in media and papain-digested cartilage samples was determined by reaction with 1,9 dimethylmethylen blue.38 Shark chondroitin sulfate7 (0 to 40 µg) was used as a standard. All samples were assayed in duplicate, and GAG content was converted to micrograms of GAG per milligram of cartilage (dry-weight basis).

Labeled GAG quantification—The amount of 35SO4-labeled GAG was quantified in papain-digested media and cartilage explants, using an Alcian Blue dye binding assay.29 This assay allows the separation of 35SO4-labeled GAG from free or unincorporated 35SO4. All samples were assayed in duplicate, and labeled GAG was converted to counts per minute per milligram of cartilage (dry-weight basis).

Measurement of total cartilage DNA content—Total DNA content was determined by a fluorometric assay, using methods reported elsewhere.28 Briefly, papain-digested cartilage explants were assayed in duplicate, and a standard curve was simultaneously generated, using DNA from calf thymus.18 Enhancement of dye fluorescence was specific for DNA, as indicated by sensitivity to DNAase and resistance to RNase digestions.8 Data were converted to DNA content per milligram of cartilage (dry-weight basis).

Determination of DNA synthesis—Synthesis of cartilage DNA was quantified by tricarboxylic acid (TCA)-precipitation of H DNA. A 50-ml aliquot of the papain-digested cartilage was added to 200 µl of a 5% TCA solution (made up in a 3:1 mixture of 100% ethanol:ethyl ether) in a single well of a 0.45-µm pore membrane filtration plate assembly.7 The plate was incubated at 4°C for 2 hours, and the aqueous fraction was filtered through the membrane, using a vacuum manifold apparatus. Any ‘H not incorporated in DNA was removed from the wells by washing each well 3 times with 200 µl of TCA solution, followed by vacuum filtration through the membrane. The size of the unincorporated ‘H allowed it to pass through the membrane, whereas the membrane retained ‘H incorporated into DNA. The bottom of the underside of the drain was blotted with absorbent paper, and dried. The membrane disk in each well was manually punched out into a 6-ml scintillation vial, and 500 µl of bleach solution (1:12.5 dilution of sodium hypochlorite)1 was added to each vial. The mixture was incubated at room temperature (20°C) for 1 hour. Four mil-

AJVR, Vol 61, No. 4, April 2000 437
liters of scintillation fluid was added to each vial, and scintillation counting was performed. Samples were assayed in duplicate, and data were converted to counts per minute per milligram of cartilage (dry-weight basis).

Statistical analyses—Data were analyzed, using a mixed-model ANOVA as described for a computerized statistics program. The dependent variables were total GAG released into the media, total GAG in the explants at termination of the experiment, 35SO4-labeled GAG remaining in the explants at termination of the experiment (pulse-chase before treatment), 35SO4-labeled GAG synthesis in the explants at termination of the experiment (pulse-chase after treatment), 3H-labeled DNA synthesis in the explants at termination of the experiment (pulse-chase after treatment), and total DNA content in explants at termination of the experiment. The independent variables were fixed effects (ie, pulse-chase labeling before and after treatment; treatment group). When main effects were significant (P < 0.05), specific comparisons were made by use of a least-squares method.

Results

Total and labeled GAG released into the media—Estimation of the amount of total GAG released into the media (average GAG measured at days 2, 4, 6, and 8 after treatment) indicated that less GAG was released into the media by explants treated with IL-1α plus IGF-1, compared with IL-1α alone (positive-control group). Also, amount of total GAG released into the media with IL-1α and IGF-1 were not significantly different than amounts for the negative-control group (ie, without IL-1α or IGF-1; Fig 1). More 35SO4-labeled GAG was released into the media from explants treated with IL-1α alone and IL-1α plus 10 ng of IGF-1/ml (Fig 2). All other explants treated with IL-1α plus IGF-1 did not differ significantly in the amounts released from those for the negative-control group, and they typically had a pattern of decreasing release with increasing IGF-1 dose, compared with that for the positive-control group, which suggested an IL-1α-induced GAG release that was partially inhibited by IGF-1 treatment.

Total GAG remaining in the explants—Explants in the positive-control group had significantly less total GAG content, compared with the negative-control group, at the termination of the experiment, suggesting an IL-1α-induced GAG loss (Fig 3). Explants treated with IL-1α plus IGF-1 had GAG content significantly less than that of the negative-control group. However, a dose-dependent response was observed, with explants treated with 500 ng of IGF-1/ml having significantly higher GAG content, compared with that for the positive-control group. Analysis of these data suggested that IGF-1 at a concentration of 500 ng/ml had a positive effect on inhibition of IL-1α-induced GAG depletion.

Pulse-chase labeling—Treatment with IL-1α (positive-control group) did not increase degradation of labeled GAG in the explants at termination of the experiment, compared with that for the negative-control group, as measured by pulse-chase labeling before treatment (Fig 4). A significant increase in retention of labeled GAG was documented with IGF-1 doses of...
50 to 500 ng/ml, suggestive of less degradation of labeled GAG. Treatment with IL-1α (positive-control group) significantly decreased GAG synthesis, compared with that for the negative-control group, as measured by pulse-chase labeling after treatment (Fig 5). Treatment with IGF-1 revealed a pattern of inhibiting this decreased synthesis in a dose-dependent manner, although values were not significantly different. Analysis of these results suggested an inhibition of GAG synthesis associated with IL-1α that may be attenuated by doses of > 500 ng of IGF-1/ml.

**Total DNA and DNA synthesis in the explants—** Only explants treated with IL-1α plus 200 ng of IGF-1/ml had a significant increase in total DNA content, compared with results for all other treatment groups (Fig 6). Explants treated with IL-1α plus 500 ng of IGF-1/ml had a higher DNA content, but not significantly so, compared with values for other treatment groups. Treatment with IL-1α significantly inhibited DNA synthesis, compared with that for the negative-control group, as measured by incorporation of 3H-thymidine (Fig 7). This effect was attenuated by treatment with IGF-1 at doses of > 200 ng/ml. Treatment with IL-1α plus 200 ng of IGF-1/ml caused significantly higher DNA synthesis, compared with that for the positive-control group. A similar pattern was observed for treatment with IL-1α plus 500 ng of IGF-1/ml, but values for that treatment did not differ significantly from those of the positive-control group. Analysis of these results suggested that IL-1α inhibited DNA synthesis and that IGF-1 concentrations of > 200 ng/ml can potentially minimize this effect.

**Discussion**

Treatment with IL-1α consistently caused cartilage matrix depletion and suppressed the mitogenic response, supporting use of IL-1α on explants as an in vitro model mimicking changes that are apparent in OA. Response of chondrocytes to IL-1α and IGF-1 was reflected in matrix GAG content, proteoglycan synthesis measured by incorporation of 35SO4 into GAG (pulse-chase labeling after treatment), proteoglycan degradation measured by loss of 35SO4-labeled GAG in the explants and media (pulse-chase labeling before treatment), and total DNA and its synthesis measured by incorporation of 3H-thymidine. In naturally developing OA, it is generally accepted that catabolic activity is mediated through contributions from systemic factors, causing direct effects on synovium, synovial fluid, and cartilage matrix and through chondrocyte- and synovioyte-mediated degradation from release or synthesis of degradative agents such as IL-1α.22-24 In vitro models have used IL-1α to induce OA conditions.14,17,25-29 However, other studies have revealed that the response of normal cartilage incubated with IL-1α does not always correlate to that for cultured cartilage obtained from an animal with naturally developing OA, especially when compared on the basis of responsiveness to IGF-1.15 Recently, investigators have suggested that a nonresponsive state may be attributable to increased local production of IGF-binding proteins in the presence of IL-1α.8 In that study, investigators used only low concentrations of IGF-1 (0 to 50 ng/ml) and high concentrations of IL-1α (1,000 ng/ml) and did not consider the potential effects that might have been obtained by use of higher concentrations of IGF-1. On the basis of results of the study reported here, a higher dose of IGF-1 (in the range of 500 ng/ml) may have yielded other results for that other study.8 Because it is not possible to regulate all of the factors in OA, we chose the in vitro model of OA (ie, created by treatment of explants with IL-1α) in an attempt to assess the impact of IGF-1 at doses ranging from 0 to 500 ng/ml on IL-1α-mediated chondrocyte anabolic and catabolic processes, realizing this may not correlate to all other models of OA.

Interleukin-1α at a concentration of 40 mg/ml caused approximately a 2-fold increase in release of labeled GAG into the media, decreased the total GAG content in the explants to approximately a fourth, decreased GAG synthesis by approximately half, and decreased DNA synthesis to approximately a fourth.
These results are similar to those of other studies that used explants from horses and other species.\textsuperscript{1,4,16,31-33} Overall, IGF-1 seemed to be associated with a protective effect on the cartilage explants. A decrease in the amounts of total and labeled GAG released into the media, compared with that for explants treated with IL-1\(\alpha\) (positive-control group) and maintenance of these 2 factors at values that were not significantly different from that for the negative-control explants, supported this premise. Approximately a 2-fold increase in total and newly synthesized GAG in the explants at termination of the experiment was observed after treatment with 500 ng of IGF-1/ml, although values were still significantly less than those of the negative-control group. Amounts of labeled GAG remaining in the explants were at least 2-fold higher for explants treated at IGF-1 concentrations of 50 ng/ml when compared with values for the positive-control group. When the mean amount of labeled GAG released into the media was compared with the amount of labeled GAG retained in the explants at termination of the experiment, some contradictions were evident because of averaging over the experimental period. Specifically, a greater amount of labeled GAG was retained in some explants treated with IL-1\(\alpha\) plus IGF-1 in the face of a greater amount of labeled GAG that was apparently being released into the media, on average, for similarly treated groups. This discrepancy can be explained when the amount of labeled GAG released into the media is evaluated at each measured time point (ie, every 2 days). Greater amounts of labeled GAG were released into the media from explants treated with IGF-1 (50 to 500 ng/ml) during the first 4 days of culture, whereas smaller amounts of labeled GAG were released into the media during the remainder of the experimental period for similar treatment groups, compared with that for the explants in the negative-control group (data not shown). This phenomenon may have been attributable to a more immediate response to the addition of IL-1\(\alpha\) and a delayed response to IGF-1 treatment.

Concentrations of IGF-1 of 200 ng/ml attenuated the actions of IL-1\(\alpha\) and restored DNA synthesis to values consistent with those of the negative-control group. These results confirm those of other studies that documented the ability of IGF-1 to combat the detrimental effects of IL-1\(\alpha\) on chondrocyte-mediated matrix metabolism.\textsuperscript{4,16,31-33} When IGF-1 was combined with an anti-inflammatory agent,\textsuperscript{4} similar effects of IGF-1 were achieved in vivo, using dogs in a model of OA, as evidenced by Mankin scores and the fact that neutral metalloproteinases, total collagenase, uronate, and hydroxypyroline contents were at approximately normal values, further supporting the use of IGF-1 for treatment of cartilage diseases. An exact mechanism by which the effects of IGF-1 are obtained is unclear. There is evidence that regulation at the receptor level does contribute to biological effects associated with IGF-1.\textsuperscript{3,16,33} Also, by using anticytokine antibodies (IL-1\(\alpha\), IL-1\(\beta\), IL-1-receptor antagonist, tumor necrosis factor), changes in the response of cartilage to IGF-1 were not detected,\textsuperscript{14} suggesting that IGF-1 may be acting directly on chondrocytes without secondary effects on cytokines that typically are associated with mediation of joint disease. Additional studies on mechanisms of action of IGF-1 on chondrocytes need to be performed.

The doses of IGF-1 for this experiment were chosen on the basis of a range that was at least 250 ng/ml greater than for other reports.\textsuperscript{4,14,16,31-33} This range was chosen to determine whether there was a plateau to the IGF-1 dose response. An expected dose response was seen with increasing concentrations of IGF-1; however, at 500 ng/ml, a plateau in the responses was not always evident, leading the authors to recommend that an IGF-1 concentration of at least 500 ng/ml be used in future studies.

Despite the positive effects of IGF-1 in the study reported here and other studies,\textsuperscript{6,9,36,37} other investigators have described a nonresponsive state to IGF-1 treatment in OA cartilage, as measured by GAG synthesis.\textsuperscript{1,14} However, as mentioned, this nonresponsive state was overcome with the addition of triamcinolone acetonide, and various, especially higher, concentrations of IGF-1 were not evaluated. The ability of triamcinolone to inhibit nonresponse to IGF-1 in one study\textsuperscript{30} suggests that an inflammatory mediator may be responsible for the nonresponsive state and may be overcome with combination treatments. Additional studies are needed to determine a dose-response effect of triamcinolone when combined with IGF-1 treatment on cultured OA cartilage explants.

\textsuperscript{1}Gibco-Life Technologies, Grand Island, NY.
\textsuperscript{2}Sigma Chemical Co, St Louis, Mo.
\textsuperscript{3}Millipore, Bedford, Mass.

\textbf{References}

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