Effects of equine recombinant interleukin-1α and interleukin-1β on proteoglycan metabolism and prostaglandin E_2 synthesis in equine articular cartilage explants

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Objectives—To evaluate the effects of equine recombinant interleukin-1α (rEqIL-1α) and recombinant interleukin-1β (rEqIL-1β) on proteoglycan metabolism and prostaglandin E_2 (PGE_2) synthesis by equine articular chondrocytes in explant culture.

Sample Population—Near full-thickness articular cartilage explants (approx 50 mg) harvested from stifle joints of a 3-year-old and a 5-year-old horse.

Procedure—Expression constructs containing cDNA sequences encoding EqIL-1α and EqIL-1β were generated, prokaryotically expressed, and the recombinant protein purified. Near full-thickness articular cartilage explants (approx 50 mg) harvested from stifle joints of a 3-year-old and a 5-year-old horse were separately randomized to receive rEqIL-1α or rEqIL-1β treatments (0 to 500 ng/ml). Proteoglycan release was evaluated by 1,9-dimethylmethylene blue spectrophotometric analysis of explant media glycosaminoglycan (GAG) concentration and release of ^{35}S-sulfate-labeled GAG to explant media. Proteoglycan synthesis was assessed by quantification of ^{35}S-sulfate incorporation into proteoglycan. Explant media PGE_2 concentrations were evaluated using a PGE_2-specific enzyme-linked immunoassay. Data were collected at 48-hour intervals and normalized by DNA content.

Results—Proteoglycan release was induced by rEqIL-1α and rEqIL-1β at concentrations ≥ 0.1 ng/ml, with 38 to 76% and 88 to 98% of total GAG released by 4 and 6 days, respectively. Inhibition of proteoglycan synthesis (42 to 64%) was observed at IL-1 concentrations ≥ 0.1 ng/ml at 2 and 4 days. Increased PGE_2 concentrations were observed at IL-1 concentrations ≥ 0.1 ng/ml at 2 and 4 days.

Conclusions and Clinical Relevance—The rEqIL-1 induced potent concentration-dependent derangement of equine chondrocyte metabolism in vitro. These findings suggest this model may be suitable for the in vitro study of the pathogenesis and treatment of joint disease in horses. (*Am J Vet Res* 2002; 63:551–558)

Interleukin-1 (IL-1) cytokines have been widely studied for their role in the regulation of inflammatory and immunologic responses to injury and disease.[1,2] The 2 agonist members of the IL-1 family, IL-1α and IL-1β, have been shown to evoke signal transduction in response to binding only a few transmembrane receptors (ie, IL-1RI) per cell and to induce their cell-type dependent and pleiotropic effects at picomolar and fentomolar concentrations.[1]

Interleukin-1 mediates a number of pro-inflammatory and early pathogenic responses thought to be involved in the initiation of joint disease in man and other animals,[2-8] including the induction[9] and synthesis[10] of phospholipase A_2, increased synthesis of cyclooxygenase and **prostaglandin E_2 (PGE_2)**, and thromboxane B_2.[11,12] Additionally, IL-1 has been shown to increase the production of tissue plasminogen activator, urinary plasminogen activator,[13,14] and nitric oxide synthase,[15] induce a dose-dependent release of extracellular cartilage matrix **proteoglycans (PG)**,[4,16,17] inhibit PG synthesis by articular chondrocytes,[18] and inhibit the synthesis of the hyaline cartilage specific collagen types II, IX, and XI.[19] **Recombinant IL-1 (rIL-1)** have been shown to induce cartilage degradation in association with the increased induction[20,21] and secretion of zinc-dependent matrix metalloproteinase by chondrocytes and synoviocytes.[5,22,23] It has been suggested that during naturally developing **osteoarthritis (OA)**, an imbalance develops in homeostatic degradative and repair mechanisms, ultimately resulting in the progressive and irreversible destruction of the articular cartilage.[24-27]

Results of in vivo[28,29] and in vitro[30-35] studies have convincingly supported the hypothesis that IL-1 may play an important role in the pathogenesis of OA in horses. High synovial fluid concentrations of IL-1 were found in association with spontaneously developing OA in horses.[28] High **glycosaminoglycan (GAG)** concentrations were also detected in the synovial fluid, serum, and urine of horses with OA relative to clinically normal horses.[29] Results of previous investigation have indicated that the effects of human rIL-1 on equine articular cartilage in explant culture include the inhibition of PG synthesis[31-35] and induction of PG

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degradation.[33,34,36] Concentrations of IL-1 necessary to elicit these effects on PG metabolism in vitro have varied. Results of 1 study[32] suggested that greater concentrations of IL-1 were necessary for the induction of PG degradation compared with those resulting in the inhibition of PG synthesis. Results of another study[35] suggested that PG synthesis was inhibited to a greater extent following treatment of equine articular cartilage explants with human rIL-1β, compared with human rIL-1α, although differences in bioactivity of the 2 proteins was not detected by an EL-4/CTLL murine lymphocyte bioassay.

The objectives of the study presented here were to characterize the expression and purification of **equine rIL-1α (rEqIL-1α)** and **equine rIL-1β (rEqIL-1β)**[37] and to determine their effects on PG metabolism and PGE_2 production by equine articular cartilage in explant culture. Additional objectives included the study of the qualitative and quantitative differences between rEqIL-1α– and rEqIl-1β–induced effects and the characterization of appropriate conditions for use of these species-homologous recombinant proteins in a rEqIL-1 equine articular cartilage explant model suitable for the study of the pathogenesis and treatment of joint disease in horses.

Materials and Methods

Expression construct design—Protein expression constructs for the production of the putative mature forms of rEqIL-1α and rEqIL-1β were generated by the polymerase chain reaction, using previously described methods.[38,a] The templates used were the full-length cDNA for rEqIL-1α and rEqIL-1β (pBluescript SK(+/-)/EqIL-1).[37] Synthetic primers were designed with overhangs producing *BamH1* and *HindIII* restriction enzyme sites and bracketing the predicted mature forms of the proteins: S-113 to F-270 for rEqIL-1α and A-116 to A-268 for rEqIL-1β.[37] A nucleotide sequence encoding a pentapeptide enterokinase cleavage site (D_4K) was placed immediately 5' to the amino terminus of the mature proteins (**Fig 1**). The amplification products were cloned into pQE-30[a] encoding a hexahistidyl peptide at the amino terminus of the translated fusion protein. Competent M15[pREP4][b] host cells were transformed with pQE-30/EqIL-1α and pQE-30/EqIL-1β, and the nucleotide sequence of transformants was verified, using the Sanger dideoxy chain termination technique.[39]

Expression and purification of rEqIL-1—The M15[pREP4] host cells transformed with the described pQE/EqIL-1α and pQE/EqIL-1β constructs were cultured in 1 L of Luria Bertani media. Expression of the recombinant proteins was induced by the addition of isopropyl β-D-thiogalactopyranoside (2 U/L). Recovered cells were lysed by sonication and lysates were loaded onto affinity chromatography columns containing a slurry of nickel nitrilo-triacetic acid resin.[b] The columns were washed with unbound proteins and eluted with a 0.3*M* imidazole solution. The fusion proteins were subjected to enterokinase[c] digestion (approx 7 U/ml) for 24 hours at 4 C and dialyzed overnight against 2 changes of PBS solution at 4 C. Digest solutions (approx 5 ml) were subjected to size exclusion fast protein liquid chromatography,[d] using a PBS running buffer at a 0.75 ml/min flow rate at 4 C. The final protein solutions were incubated with an endotoxin removal protein-linked resin[e] for 24 hours at 4 C and separated by centrifugation.

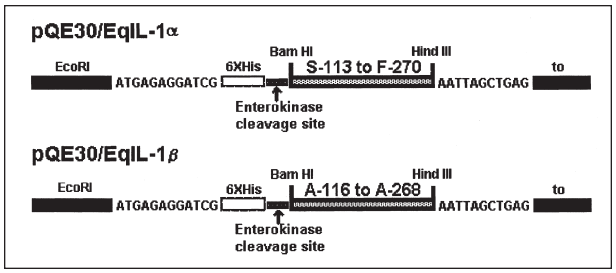

Figure 1—Diagram of equine recombinant interleukin-1 (rEqIL-1) protein constructs.

Protein analysis—Protein purity and electrophoretic migration were evaluated by sodium dodecyl-sulfate polyacrylamide gel electrophoresis. Single protein band fast protein liquid chromatography fractions, as detected by Coomassie blue staining and with electrophoretic migration consistent with the mature forms of rEqIL-1, were pooled. Protein purity was further characterized by high-pressure reversed phase liquid chromatography.[f] The concentration of endotoxin in purified protein solutions was determined, using an assay.[e] Final protein concentrations were determined, using the Bradford spectrophotometric assay.[40]

Bioactivity of rEqIL-1—Recombinant protein bioactivity was determined, using a previously described murine T-cell mitogenesis bioassay.[41] Briefly, D10(N4)M murine T-lymphocytes[g] were cultured in RPMI 1640[h] supplemented with 10% fetal bovine serum,[g] 0.02*M* β-mercaptoethanol, concanavalin A (3 μg/ml), murine rIL-1β[h] (8 U/ml), human rIL-2[h] (20 U/ml), and 10% penicillin-streptomycin at 37 C, 5% CO_2. Cells in log phase growth were plated in quadruplicate at 1×10^4 cells/well in 96-well plates with serial dilutions of rIL-1 treated media. Spectrophotometric absorption was measured at 570 and 600 nm (116 hours) following the addition of an alamar blue dye solution.[i] The difference in absorbances at 570 and 600 nm, corresponding to the amounts of the reduced and oxidized forms of the dye respectively, were used as the estimate of mitogenesis:

$$\text{Absorbance}_{\text{final}} = A_{570} - A_{600}$$

The mean oxidative responses to rEqIL-1 were plotted as functions of log dilutions relative to a commercially available murine rIL-1β[h] (1×10^7 U/mg).

Explant culture conditions—Two independent experiments were conducted to test the effects of rEqIL-1 on equine articular cartilage in explant culture. Near full-thickness articular cartilage was aseptically removed from the stifle joints of a 3-year-old horse (experiment 1) and a 5-year-old horse (experiment 2) euthanatized for reasons unrelated to joint disease at the Virginia-Maryland Regional College of Veterinary Medicine. Cartilage for each experiment was pooled in sterile Gey balanced salt solution,[h] cut into explants of similar wet weight (approx 50 mg), and randomly assigned to 24-well plates. Explants were equilibrated for 48 hours at 37 C, 5% CO_2 in 2 ml of a modified culturing media consisting of high-glucose Dulbecco medium[h] supplemented with 10% fetal bovine serum,[h] 0.24 m*M* ascorbic acid, 1.78 m*M* L-glutamine, 0.14 m*M* α-ketoglutaric acid, 21.5 m*M* HEPES, and penicillin (57 U/ml)-streptomycin (57 μg/ml).[42] Experimental wells were replaced with media containing rEqIL-1α or rEqIL-1β at 0.1, 1.0, 10, 50, 100, and 500 ng/ml concentrations over a 6-day treatment period (experiment 1). The sample collection protocol of experiment 2 was designed to evaluate the effects of rEqIL-1 over a lower range of concentrations and for a shorter duration of exposure: 0.01, 0.1, 1.0, 10, and 100 ng/ml concentrations over a 4-day treatment period. Media was harvested and replaced at 48-hour inter-

vals. Final media samples and explants used to evaluate ^{35}S-sulfate and sulfated GAG release were harvested on day 6 (experiment 1) and on day 4 (experiment 2). Explants were harvested on days 2 and 4 (experiment 1) and on day 2 (experiment 2) for the evaluation of ^{35}S-sulfate incorporation. Control explants for both experiments were treated with supplemented media lacking rIL-1. All explant conditions were conducted in quadruplicate for each experiment. Harvested samples were frozen at –20 C until bulk analysis.

Spectrophotometric analysis—Harvested media samples were incubated at 65 C for 4 hours with an equal volume of papain[j] (0.5 mg/ml) in digest buffer (50 m*M* $NaPO_4$, 2 m*M* N-acetyl cysteine, 4 m*M* EDTA disodium salt, pH 6.5). Harvested explants were incubated at 65 C for 4 hours in digest buffer (1 ml/10 mg wet weight) containing papain (0.5 mg/ml). Aliquots of digested media (200 µl) and explants (50 µl) were analyzed for GAG content, using a modification of a **1,9-dimethylmethylene blue**[k] **(DMMB)** labeling technique.[43] Sulfated GAG concentrations were determined by absorbance readings at 525 nm relative to a shark chondroitin-4-sulfate standard curve (0 to 50 µg/ml). Each sample was analyzed in triplicate. The GAG release data for both experiments were expressed as normalized raw values as well as proportions of available explant GAG released to the media as follows (eg, for Day 2):

$$\text{Day 2 GAG release} = \frac{\text{Day 2 GAG}}{\text{Days 2 + 4 + 6 + explant GAG}}$$

Additionally, cumulative GAG release data for both experiments were expressed as proportions of the total available GAG over the experimental periods as follows:

$$\text{Cumulative GAG release} = \frac{\text{Days 2 + 4 + 6 GAG}}{\text{Days 2 + 4 + 6 + explant GAG}}$$

^{35}S-sulfate release—Explants were incubated with ^{35}S-sulfate labeled $NaSO_4$ (20 µCi/ml) for 48 hours prior to the addition of rEqIL-1. Harvested media and explant samples were papain-digested, using the conditions previously described for the DMMB spectrophotometric analysis. Aliquots (25 µl) of papain-digested cartilage and media samples were added to 96-well 0.45-µm filter-bottomed plates[l] and precipitated, using an Alcian blue dye solution (0.2% [wt/vol] Alcian blue,[h] 0.05*M* sodium acetate, 0.085*M* $MgCl_2$). The precipitate was recovered by vacuum filtration and a multiwell punch system.[l] Emission counts were recorded, using a beta-scintillation counter. Each sample was analyzed in triplicate. Data were expressed as normalized raw values and as proportional ^{35}S-sulfate released to the media at each point of media harvest and over the experimental period combined, as described for the DMMB analysis.

^{35}S-incorporation—Explants were incubated with ^{35}S-sulfate labeled $NaSO_4$ (20 µCi/ml) for 16 hours prior to explant harvest on day 2 and day 4 (experiment 1) and on day 2 (experiment 2). Explant digestion, GAG precipitation, and recovery for scintillation counting was conducted as previously described. Each sample was analyzed in triplicate.

Deoxyribonucleic acid quantitation—Explant DNA concentrations were evaluated, using a previously reported technique.[44] Briefly, aliquots of papain-digested cartilage samples (100 µl) were diluted in a 0.1 µg/ml dye[m] solution. Spectrofluorometry was performed, using an excitation wavelength of 365 nm and emission detection at 458 nm. Explant DNA concentrations were determined relative to a calf thymus DNA standard curve (0 to 100 µg/ml). Explant analyses were conducted in triplicate.

PGE_2 enzyme immunoassay—Release of PGE_2 to the media at days 2 and 4 (experiment 2) were quantitated, using a commercially available enzyme-immunoassay kit[n] based on the competitive binding of PGE_2-specific antibody with a fixed quantity of peroxidase-conjugated PGE_2. Media samples (50 µl) from duplicate wells for control and rEqIL-1 treatment conditions (0 to 100 ng/ml) were diluted in the provided assay buffer. Absorbance was read at 450 nm, and PGE_2 concentrations (pg/ml) were deduced by comparison with a standard curve (50 to 6,400 pg of PGE_2/ml).

Results

Protein purification—The yield of purified rEqIL-1α and rEqIL-1β from 1 L cultures was 0.093 and 3.80 mg, respectively, representing an approximate 40-fold greater yield of purified rEqIL-1β relative to rEqIL-1α. Final protein concentrations for rEqIL-1α and rEqIL-1β were 4.24 and 70.75 µg/ml, respectively. Purified rEqIL-1α and rEqIL-1β had electrophoretic migration patterns on sodium dodecyl-sulfate polyacrylamide gel electrophoresis consistent with that expected for the mature form of the proteins. Protein purity was estimated at near 100% for rEqIL-1α and rEqIL-1β, as determined by the detection of single peaks on high-pressure reversed-phase liquid chromatography. Endotoxin concentrations were determined to be ≤ 0.01 ng/µg of protein. The bioactivity of rEqIL-1α and rEqIL-1β was 6.6×10^6 and 4.7×10^6 U/mg, respectively.

DMMB spectrophotometric analysis—Media GAG concentrations were increased in rEqIL-1α– and rEqIL-1β–treated explants (0.1 to 500 ng/ml), compared with untreated control explants at day 2 (298 to 500%), day 4 (188 to 463%), and day 6 (86 to 200%; **Fig 2a**). The proportions of available GAG released to the explant media in response to rEqIL-1 (0.1 to 500 ng/ml) were increased at days 2, 4, and 6 (**Fig 2b**). Over a cumulative 4-day treatment, the mean cumulative release of GAG from explants exposed to rEqIL-1 (0.1 to 100 ng/ml) was 38 to 67%. In explants treated with 0.01 ng of rEqIL-1/ml the mean cumulative release of GAG was 18%; it was 10% in the untreated control explants. Over a cumulative 6-day treatment, a

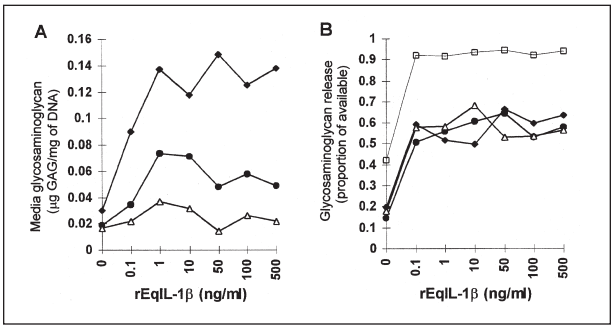

Figure 2—Results of 1,9-dimethylmethylene blue (DMMB)-spectrophotometric analysis: Panel A—Raw normalized sulfated GAG concentrations detected in the media of equine articular cartilage explants exposed to rEqIL-1β (0 to 500 ng/ml) at 2, 4, and 6 days (experiment 1). Panel B—Proportions of available GAG released to explant media in response to rEqIL-1β (0 to 500 ng/ml; experiment 1). Data points represent the mean values of quadruplicate wells normalized for DNA content and reflect the effects of rEqIL-1α. Closed diamond = Day 2. Closed circle = Day 4. Open triangle = Day 6. Open square = Cumulative release of available GAG on days 2, 4, and 6.

mean of 88 to 94% of the total GAG was released to the media in rEqIL-1–treated explants (0.1 to 500 ng/ml) relative to a mean of 42% for control explants. Effects on GAG release as detected by this analysis were similar for rEqIL-1α and rEqIL-1β.

^{35}S-sulfate release—Increased amounts of ^{35}S-sulfate-labeled GAG were detected in the media from rEqIL-1α– and rEqIL-1β–treated explants (0.1 to 500 ng/ml) relative to untreated control explants at day 2 (1,011 to 2,082%), day 4 (558 to 977%), and day 6 (203 to 533%). The proportions of available ^{35}S-sulfate-labeled GAG released in response to rEqIL-1 (0.1 to 500 ng/ml) were greater than control explant proportional GAG release. Similar responses were observed at days 2, 4, and 6. Over a cumulative 4 day treatment, 50 to 76% of the total available ^{35}S-sulfate-labeled GAG was released to the explant media in the rEqIL-1–treated explants (0.1 to 500 ng/ml), compared with a mean of 34% in explants treated with 0.01 ng of rEqIL-1/ml and a mean of 17% for the untreated control explants (**Fig 3**). Over a cumulative 6-day treatment, 90 to 98% of the total labeled GAG was released to the media in rEqIL-1 treated explants (0.1 to 500 ng/ml) compared with the mean release of 37% in control explants. The effects on ^{35}S-sulfate release were similar for rEqIL-α and rEqIL-1β.

^{35}S-sulfate-incorporation—The incorporation of ^{35}S-sulfate was decreased in rEqIL-1α– and rEqIL-1β–treated explants (0.1 to 500 ng/ml) harvested at day 2 and at day 4 relative to untreated control explants. Similarly, rEqIL-1 treated explants (0.01 to 100 ng/ml) exhibited decreased ^{35}S-sulfate incorporation at day 2 (**Fig 4**). Inhibition of ^{35}S-sulfate incorporation was less apparent at 0.01 ng of rEqIL-1/ml. The effects of rEqIL-1 on ^{35}S-sulfate incorporation were similar at day 2, compared with day 4. The mean inhibition of ^{35}S-sulfate incorporation associated with rEqIL-1α and rEqIL-1β treatments (0.01 to 500 ng/ml) was ≥ 53 and ≥ 47%, respectively, at day 2 and ≥ 51 and ≥ 59% at day 4 for rEqIL-1α and rEqIL-1β (0.1 to 500

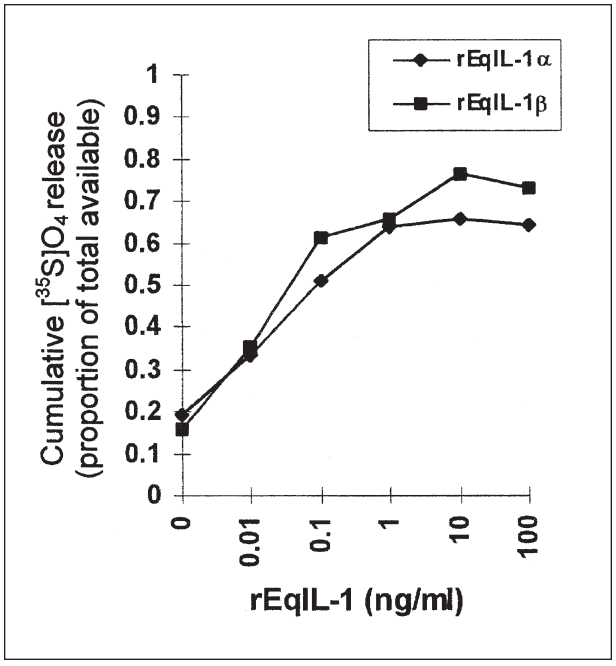

Figure 3—Cumulative release of ^{35}S-sulfate-labeled GAG to explant media from equine articular cartilage explants treated with rEqIL-1α and rEqIL-1β (0 to 100 ng/ml) over a 4-day period (experiment 2). Data are expressed as the proportions of total available ^{35}S-sulfate-labeled GAG released to the media (ie, days 2 + 4 media cpm/days 2 + 4 explant cpm). Data points represent the mean values of quadruplicate wells normalized for DNA content.

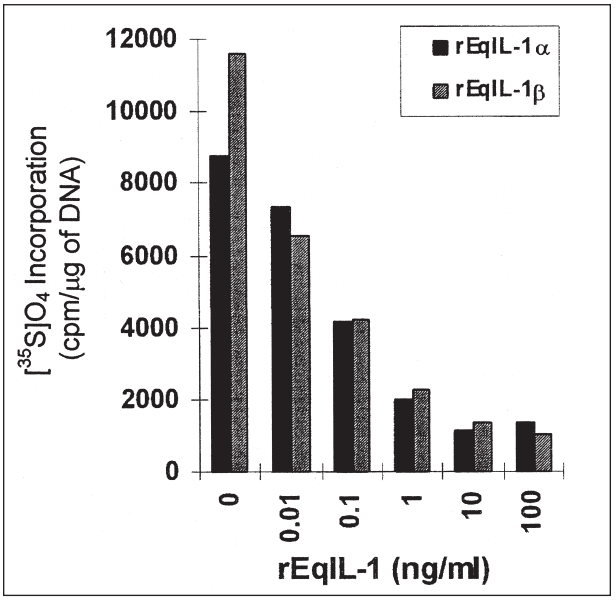

Figure 4—Effects of rEqIL-1α and rEqIL-1β (0 to 100 ng/ml) on proteoglycan (PG) synthesis, as determined by ^{35}S-sulfate incorporation in equine articular cartilage explants at day 2 (experiment 2). Data points represent the mean values of quadruplicate wells normalized for DNA content.

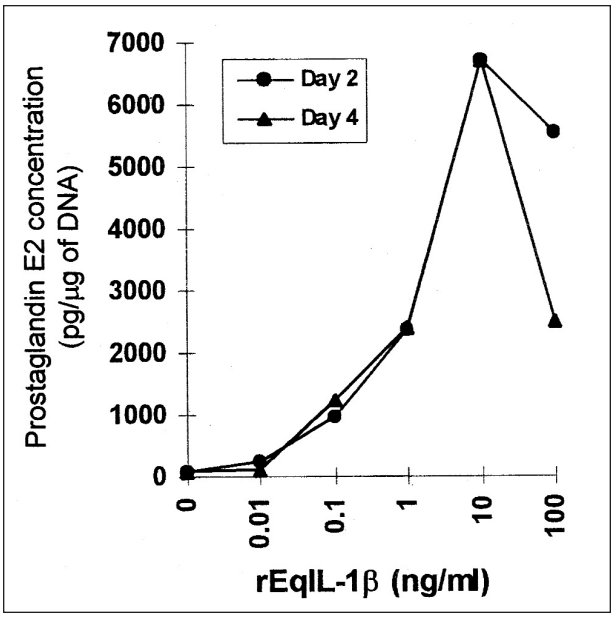

Figure 5—Prostaglandin E_2 (PGE_2) concentration in conditioned media from equine articular cartilage explants at days 2 and 4 (experiment 2) in response to rEqIL-1β (0 to 100 ng/ml), as determined by enzyme immunoassay analysis. Data points represent the mean values of duplicate wells normalized for DNA content and reflect the effects of rEqIL-1α.

ng/ml), respectively. Inhibition of ^{35}S-sulfate incorporation was similar for rEqIL-1α and rEqIL-1β.

PGE_2 assay—Both rEqIL-1α and rEqIL-1β (0.1 to 100 ng/ml) induced a dose-dependent increase in PGE_2 concentrations in explant media at day 2 and day 4 relative to untreated control explants (**Fig 5**). Induced effects were not as apparent at the 0.01-ng/ml concentrations for rEqIL-1α or rEqIL-1β at day 2 or day 4 evaluations. Explant media PGE_2 concentrations were similar for rEqIL-1α– and rEqIL-1β–treated explants.

Deoxyribonucleic acid assay—Mean DNA concentrations of papain-digested control and rEqIL-1–treated explants (0.01 to 100 ng/ml) harvested at days 2 and 4 and rEqIL-1–treated explants (0.1 to 500 ng/ml) harvested at days 2, 4, and 6 were similar and not affected by rEqIL-1 exposure or duration of culture.

Discussion

The results of our study suggest that rEqIL-1α and rEqIL-1β induced a dose-dependent derangement in proteoglycan metabolism and induced the synthesis of PGE_2 in equine articular cartilage in explant culture. These results suggest that the rEqIL-1/equine articular cartilage explant system may be a useful model for the in vitro study of the pathogenesis and treatment of joint disease in horses. Our findings are in general agreement with those of investigators who have demonstrated human rIL-1 to induce PG degradation[33,34,36] or to inhibit PG synthesis[31-35] in equine cartilage explants. Near maximal responses for PG degradation and inhibition of PG synthesis were observed at rEqIL-1 concentrations ≥ 0.1 ng/ml. The results of our study differ from those of previous studies in that substantial effects on PG degradation or synthesis were observed at rEqIL-1 concentrations 40 to 100 times lower than the lowest human rIL-1 treatment concentrations reported to induce these effects, ranging from 4 to 10 ng/ml.[31,33-35] Additionally, the results of our study differ from a previous report[32] suggesting inhibition of proteoglycan synthesis developed at lower concentrations of human rIL-1 than concentrations resulting in substantial PG degradation in equine articular cartilage explants. Our study was not designed to address the differences in effects on PG metabolism in equine articular cartilage explants associated with the use of human rIL-1 or rEqIL-1. Whether these potential differences had a biological basis or were reflections of different investigators and methods remains to be determined. However, our results would indicate lower concentrations of rEqIL-1 should be considered for equine articular cartilage explant studies, compared with those previously reported in studies using human rIL-1.

It has been suggested that the biological response to IL-1 is to some extent species-specific.[45,46] The results of 1 study[46] highlighted the preferential stimulation of bovine thymocytes and fibroblasts by bovine IL-1, compared with human rIL-1. Results of another study[45] indicate that equine thymocytes had increased sensitivity and proliferation to an equine mononuclear cell supernatant, compared with human rIL-1. It has been proposed that a species-homologous equine IL-1 would be necessary to directly address the elucidation of the effects of IL-1 on chondrocyte metabolism in an equine system.[45] The observation of these investigators may be attributable to crucial differences in the amino acid sequences of equine and human IL-1, with amino acid sequence homology previously reported as 66.4%[37] and 71.6%[47] for IL-1α and 66.7%[47] and 72.7%[37] for IL-1β. These sequence differences may in turn confer changes in receptor binding and activation. Our results indicate that rEqIL-1 have potent effects on equine articular cartilage metabolism and would appear to support the use of species-homologous IL-1 in such in vitro studies. Further experiments are required to address the issue of species-specificity directly.

In our study, evaluation of the raw normalized data suggested the magnitude of GAG released to the explant media, as determined by DMMB spectrophotometry and the release of ^{35}S-sulfate-labeled GAG, was influenced by the duration of exposure to rEqIL-1. Evaluation of these data alone may suggest a loss of responsiveness over time to rEqIL-1. The magnitude of release of GAG would logically depend on the amount of degradative activity by chondrocytes as well as the availability of substrate (ie, PG). Evaluation of the proportion of available GAG released to the explant media indicated that the release of GAG was similar over all time points evaluated. Approximately 75% of the total GAG had been released by day 4, and 90% had been released by day 6. We conclude that the lack of effect evidenced by the day 6 raw normalized GAG release data is attributable to substrate depletion rather than a lack of chondrocyte rEqIL-1 responsiveness.

The results of our study indicate that the prokaryotic expression and native purification of rEqIL-1α and rEqIL-1β provided an acceptable yield of purified recombinant proteins exhibiting authentic IL-1 bioactivity. The yield of rEqIL-1β in our study exceeded that of rEqIL-1α by approximately 40-fold. This difference in protein yield was not the result of differences in expression (data not shown) but was attributable to the decreased aqueous solubility of rEqIL-1α, compared with rEqIL-1β. Methods of purification using denaturing conditions would likely produce improved and similar yields of rEqIL-1α and rEqIL-1β. Purification under denaturing conditions requires accurate refolding of the protein for the restoration of the bioactivity. The yields and bioactivity, using the described methods, were acceptable, and the potential difficulty associated with renaturation of recombinant proteins was avoided.

It was important to ensure that the purified rEqIL-1 proteins contained acceptable amounts of **lipopolysaccharide (LPS)**, because LPS has been shown to induce derangements in PG metabolism and eicosanoid production in equine articular cartilage explants that are qualitatively similar to those induced by IL-1.[48] The prokaryotic expression of recombinant protein in our study presented ample opportunity for LPS contamination derived from the host cell M15 strain of *Escherichia coli*. The described method for removal of LPS was effective in reducing the LPS concentration to ≤ 0.01 ng/μg of protein. The manufacturer of a commercially available human rIL-1° considered appropriate for tissue culture indicates its product has < 0.1 ng of LPS/μg protein,

suggesting this commercially available rIL-1 could contain up to 10 orders of magnitude more LPS than the rEqIL-1 used in our study. In an evaluation of the effects of *Salmonella* ser. Typhosa derived LPS on equine articular cartilage explants in culture, a dose-dependent decrease in PG synthesis, dose-dependent increase in GAG release, and increase in PGE_2 synthesis was demonstrated.[48] In the cited study, LPS (10 ng/ml) induced a significant reduction of PG synthesis in articular cartilage explants obtained from 10 of 15 horses, whereas the highest possible concentration of endotoxin that was associated with rEqIL-1 treatment in our study was 0.005 ng/ml. Therefore, the contribution of LPS to the observed effects of rEqIL-1 on equine articular cartilage explants presented in our study should be considered as negligible.

The D10(N4)M murine T-cell line has been previously shown to undergo mitogenesis in the presence of IL-1 with high sensitivity and specificity.[41] Although the D10(N4)M bioassay was helpful in establishing that the purified recombinant proteins qualitatively had authentic IL-1 bioactivity, the validity of the bioassay for the quantitative determination of equine IL-1 bioactivity was less certain. The bioactivity of the purified rEqIL-1α and rEqIL-1β was determined to possess 66 and 47%, respectively, the bioactivity of a murine recombinant IL-1β (1×10^7 U/mg). We did not directly determine whether the decreased responsiveness of the D10(N4)M cells to the rEqIL-1 was attributable to a relative decreased bioactivity of the proteins compared with murine IL-1β or to potential differences in responsiveness of the murine T-cells to murine and equine IL-1. Because the rEqIL-1 bioactivity was not determined using an equine cell line and the rEqIL-1 appeared to have good bioactivity on equine articular cartilage explants in a pilot study (data not presented), the treatments were applied on a mass/ml rather than bioactivity/ml basis.

This approach appeared to be reasonable, because differences were not observed in the effects induced by rEqIL-1α and rEqIL-1β, despite the indication from the D10(N4)M bioassay that the bioactivity of rEqIL-1β was 71% that of rEqIL-1α. It is unlikely that the experimental design used in our study evaluating rEqIL-1 treatments applied in orders of magnitude of concentration difference and methods of analysis used were capable of detecting the 29% difference in bioactivity predicted by the D10(N4)M bioassay. Ultimately, it was unclear whether this 29% apparent difference in bioactivity was attributable to differences in the quality of the protein preparations or was the result of biological differences in the murine cell responses to the different isoforms of rEqIL-1. We conclude that the effects on proteoglycan metabolism and PGE_2 synthesis were similar for the 2 rEqIL-1 proteins used in our study over all treatment conditions.

Because the yield of rEqIL-1β exceeded that of rEqIL-1α by 40-fold and the responses to the 2 proteins were similar, we intend to use rEqIL-1β in future related studies. This conclusion is consistent with a previous study[49] that had similar effects of human monocyte-derived IL-1α and IL-1β on cultured human connective tissue cells but conflicts with another similarly designed explant study[35] suggesting human rIL-1α induced a greater magnitude of effects relative to human rIL-1β on equine articular cartilage explants. The 2 isoforms of human IL-1 have been shown to possess crucial similarities in structural topology and to bind with similar affinities to common transmembrane IL-1 receptors.[1] To the authors' knowledge, this information is unavailable for the equine IL-1 system, but it is reasonable to presume that a similar biological relationship is in operation for the horse.

Consistent with the findings of other investigators evaluating the effects of human rIL-1 on equine articular cartilage cells,[3,30] the results of our study indicate that rEqIL-1 induced the dose-dependent synthesis and secretion of PGE_2 by articular cartilage explant chondrocytes to the culture media. Because the methods used in our study did not include the determination of the amount of PGE_2 contained within the explants, the total synthesis of PGE_2 was not evaluated. Only the portion that was released to the explant media was measured. It is unknown how determination of total PGE_2 synthesis may have affected our results.

The characteristics of the ideal in vitro model for the study of joint disease are a subject for debate. An acceptable model ideally would resemble the course of naturally developing disease and be associated with as few confounding variables as possible. Tissues from a limited number of horses were evaluated in our study for the purpose of establishing the validity of the methods described. One should be cautioned against the rigid extrapolation of the results of our study to other environments or subjects. The combined pattern of effects of rEqIL-1 on proteoglycan metabolism and PGE_2 synthesis observed in our study suggests that the rEqIL-1/equine articular cartilage explant model may be useful for the study of the pathogenesis and treatment of joint disease in horses. Alterations in PG metabolism and PGE_2 synthesis are processes that have been associated with naturally developing joint disease in horses, and ample evidence is available suggesting IL-1 may be an important effector in the pathogenesis of joint disease in horses. We suggest that the depletion of approximately 90% of the total GAG from the extracellular matrix of the cartilage explants over a period of 6 days or approximately 75% over 4 days represents a severe and acute insult. The indication that rEqIL-1 induced PG degradation even at the 0.01 ng/ml concentration applied over an experimental period of 4 days has prompted related studies to refine this in vitro system to more accurately represent the more insidious progression of joint problems in horses such as OA.

[a] Howard RD. *Cloning, sequence, and prokaryotic expression of the cDNAs for equine interleukin-1*. PhD dissertation, Department of Science, Colorado State University, Ft Collins, Colo, 1997.
[b] Qiagen Inc, Chatsworth, Calif.
[c] Invitrogen Co, San Diego, Calif.
[d] High-load 16/60 Superdex 75 column, Pharmacia Biotech Inc, Uppsala, Sweden.
[e] Limulus amebocyte lysate assay, Associates of Cape Cod Inc, Falmouth, Mass.
[f] Ultrapore C_3 column, Beckman Instruments Inc, San Ramon, Calif.
[g] Murine D10(N4)M T-cell line. Provided by Dr. Stephen Hopkins, University of Manchester Rheumatic Diseases Center, Manchester, UK.

[h]Gibco BRL, Life Technologies Inc, Grand Island, NY.
[i]Biosource International, Camarillo, Calif.
[j]Sigma Chemical Co, St Louis, Mo.
[k]Aldrich Chemical Co, Milwaukee, Wis.
[l]Multiscreen assay system, Millipore, Bedford, Mass.
[m]Hoeschst 33258, Molecular Probes, Eugene, Ore.
[n]Amersham Pharmacia Biotech Inc, Piscataway, NJ.
[o]Calbiochem-Novabiochem, San Diego, Calif.

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