

# Effects of proinflammatory cytokines on canine articular chondrocytes in a three-dimensional culture

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**Objective**—To determine the effects of interleukin (IL)-1 and tumor necrosis factor (TNF)- $\alpha$  on canine chondrocytes cultured in an agarose-based 3-dimensional (3-D) system.

**Sample Population**—Humeral head articular cartilage chondrocytes obtained from 6 adult dogs.

**Procedure**—Chondrocytes were cultured in a 3-D system for  $\leq$  12 days in serum-free medium with IL-1 $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  at concentrations of 20, 50, or 100 ng/mL. After 1, 3, 6, and 12 days, glycosaminoglycan (GAG) concentrations in 3-D constructs; nitric oxide and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentrations in media samples; and relative expressions of selected genes, including metalloproteinase (MMP)-13 and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, were evaluated. Control specimens were comprised of chondrocytes cultured without proinflammatory cytokines.

**Results**—In control 3-D constructs, GAG content was significantly higher than for all other constructs. Compared with control values, relative expressions of MMP-13, TIMP-1, and TIMP-2 genes in the IL-1 $\beta$  (50 ng/mL) group were significantly higher at day 1; at all evaluations, media concentrations of nitric oxide were significantly higher in all TNF- $\alpha$ -treated cultures; and concentrations of PGE<sub>2</sub> in media samples were significantly higher in the IL-1 $\beta$  (50 ng/mL) and IL-1 $\beta$  (100 ng/mL) groups at days 1 and 3, in the IL-1 $\beta$  (100 ng/mL) group at day 6, and in all TNF- $\alpha$  groups at days 1, 3, and 6.

**Conclusions and Clinical Relevance**—Results suggested that TNF- $\alpha$  more readily induces production of nitric oxide and PGE<sub>2</sub> by canine chondrocytes, compared with IL-1 $\beta$ . In vitro, IL-1 $\alpha$  appeared to have a minimal effect on canine chondrocytes. (*Am J Vet Res* 2005;66:1187–1196)

Osteoarthritis is frequently regarded as a noninflammatory form of arthritis. However, in the degradation of cartilage that is associated with osteoarthritis, considerable data support an important role of proinflammatory cytokines derived from joint tissues, including chondrocytes and synovio-cytes.<sup>1-4</sup> Interleukin (IL)-1 and tumor necrosis factor (TNF)- $\alpha$  are the principal cytokines linked to the catabolism of articular cartilage and to the disease process of osteoarthritis. Interleukin-1 and TNF- $\alpha$  have been reported to play critical roles in disease ini-

tiation and progression by increasing proteinase expression<sup>5</sup> and inhibiting synthesis of articular cartilage-specific type II collagen<sup>6</sup> and proteoglycan.<sup>7</sup> However, it has not yet been fully understood whether IL-1 and TNF- $\alpha$  act independently and whether a functional difference and hierarchy exists between them.

Further elucidation of the effects of these proinflammatory cytokines on chondrocytes is crucial not only for further understanding of the disease mechanisms of osteoarthritis but also for establishing effective and safe treatment modalities. Adverse effects associated with administration of biological agents that directly block IL-1 or TNF- $\alpha$  activity in humans with arthritis have been reported.<sup>8-10</sup> Because IL-1 and TNF- $\alpha$  are pleiotropic cytokines that have the potential to activate numerous signaling pathways and transcription factors, including those related to host defense mechanisms, treatments that suppress IL-1 or TNF- $\alpha$  activity may also inhibit the host immune system. Further investigation of the roles of IL-1 and TNF- $\alpha$  in the disease process of osteoarthritis may lead to the development of novel treatment strategies that are associated with fewer and less severe complications.

Moreover, although upregulation of the 2 isoforms of IL-1 (IL-1 $\alpha$  and IL-1 $\beta$ ) has been reported<sup>11</sup> in cartilage from osteoarthritic joints of humans and recombinant human IL-1 $\alpha$  or IL-1 $\beta$  has been widely used in vitro as an inducer of inflammation and degradation in experimental models of osteoarthritis, the specific role of each IL-1 isoform is not fully understood. The susceptibility of canine articular cartilage to recombinant human IL-1 $\alpha$  and IL-1 $\beta$  needs to be evaluated because it is possible that, like bovine cartilage explants,<sup>12</sup> canine cartilage tissue has a higher susceptibility to human recombinant IL-1 $\alpha$  than human recombinant IL-1 $\beta$ . The objective of the study reported here was to determine the effects of IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$  on canine chondrocytes cultured in an agarose-based 3-dimensional (3-D) system; assessments included extracellular matrix expression and accumulation and expression of catabolic factors. Our hypothesis was that the effects of these recombinant human proinflammatory cytokines on 3-D-cultured canine chondrocytes would be significantly different from one another.

## Materials and Methods

**Cell isolation and in vitro culture**—Full-thickness articular cartilage slices were aseptically obtained from the caudocentral portion of 1 humeral head of each of 6 canine

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cadavers (5 to 8 slices/humeral head) via arthrotomy performed immediately after euthanasia. The dogs (age range, 12 to 24 months) were apparently healthy and were euthanized by use of an overdose of pentobarbital and phenytoin for reasons unrelated to this study; the humeral head cartilage appeared grossly normal in all dogs.

Canine chondrocytes were isolated and cultured in monolayer for amplification of cell numbers prior to placement in a 3-D agarose gel, as previously reported.<sup>13-15</sup> At near confluency in monolayer culture, the cells were detached via trypsinization, washed in Hank's balanced salt solution, and counted and checked for viability by use of a trypan blue exclusion assay. In each sample, cell viability was > 95%. Equal volumes of 2% low-melting agarose<sup>a</sup> (gelling temperature 25 ± 5°C) in PBS solution and **double-strength RPMI 1640 (dsRPMI)** containing 20% fetal bovine serum were added to attain a cell concentration of 3 × 10<sup>6</sup> cells/mL. One milliliter of the cell suspension was added to each well of a 24-well tissue culture plate. The plates were placed in a refrigerator at 4°C for 5 minutes. After the agarose medium was gelled to form the 3-D construct, 1 mL of dsRPMI containing penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (2.5 µg/mL), ascorbate (50 µg/mL), and 10% fetal bovine serum was added to each well. The plates were incubated at 37°C with 5% carbon dioxide and 95% humidity, and the culture medium was refreshed twice per week.

After 14 days of 3-D culture, the 3-D constructs were washed (3 washes each of 5 minutes' duration) with serum-free dsRPMI and subsequently maintained in serum-free culture for as long as 12 days with or without recombinant human IL-1α, IL-1β, or TNF-α<sup>b</sup> at concentrations of 20, 50, or 100 ng/mL. The treatment groups were devised as follows: control group, 3-D constructs in conditioned medium alone; IL-1β(20) group, 3-D constructs in conditioned medium with IL-1β (20 ng/mL); IL-1β(50) group, 3-D constructs in conditioned medium with IL-1β (50 ng/mL); IL-1β(100) group, 3-D constructs in conditioned medium with IL-1β (100 ng/mL); IL-1α(20) group, 3-D constructs in conditioned medium with IL-1α (20 ng/mL); IL-1α(50) group, 3-D constructs in conditioned medium with IL-1α (50 ng/mL); IL-1α(100) group, 3-D constructs in conditioned medium with IL-1α (100 ng/mL); TNF-α(20) group, 3-D constructs in conditioned medium with TNF-α (20 ng/mL); TNF-α(50) group, 3-D constructs in conditioned medium with TNF-α (50 ng/mL); and TNF-α(100) group, 3-D constructs in conditioned medium with TNF-α (100 ng/mL). In each culture, the medium was changed every 3 days and fresh IL-1β-, IL-1α-, or TNF-α-supplemented medium was added at each time. Samples of culture media and 3-D constructs were collected after 24 hours, 3 days, 6 days, and 12 days of culture. Each collected 3-D construct was divided into 2 portions: 1 portion was weighed and stored at -20°C for subsequent evaluations for glycosaminoglycan (GAG) content, and the other portion was used for RNA extraction for **reverse-transcription polymerase chain reaction (RT-PCR)** assay. Samples of the medium from each culture were stored at -20°C for subsequent evaluations of GAG, nitrite, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentrations.

**Extracellular matrix concentrations in 3-D constructs**—Total sulfated GAG content was quantitated via **dimethylmethylene blue (DMMB)** assay.<sup>16</sup> Three-dimensional constructs were digested for approximately 14 hours at 60°C in 1 mL of papain<sup>c</sup> (0.5 mg/mL [14 U/mg]) in 20mM sodium phosphate buffer (pH, 6.8) containing 1mM EDTA. A 5-µL aliquot of the digest solution was assayed for total GAG

content by addition of 245 µL of DMMB solution and spectrophotometric determination of absorbance at 525 nm. Known concentrations of bovine tracheal chondroitin sulphate A<sup>c</sup> were used to construct standard curves. Total GAG content in the 3-D constructs was reported as micrograms of GAG per gram weight. In samples of conditioned media (5 µL), GAG concentrations were assayed by addition of 245 µL of DMMB solution and spectrophotometric determination of absorbance at 525 nm.

**RNA extraction and RT-PCR assay**—Total RNA was extracted from half of each 3-D construct via incubation for 30 seconds at 90°C in 0.5 mL of water-saturated phenol containing 50 µL of 3M sodium acetate solution (pH, 5.5), then incubated at 4°C for approximately 14 hours. The phases were separated by centrifugation at 4°C for 10 minutes; the aqueous layer was transferred into a microcentrifuge tube and desiccated by use of 1-butanol to a volume of approximately 100 µL. The sample was transferred to another tube and mixed with ethanol; the RNA was precipitated by centrifugation for 10 minutes. The RNA pellet was resuspended in 100 µL of RNase-free water, and the RNA was purified by use of a commercial kit<sup>d</sup> according to the manufacturer's protocol. The RNA was eluted in RNase-free water and quantified by absorbance at 260 nm.

Total RNA was reverse transcribed to cDNA via RT involving random hexamers,<sup>e</sup> Moloney murine leukemia virus RT,<sup>f</sup> deoxynucleotide triphosphates, and RT buffer to a final reaction volume of 20-µL reaction. Reverse transcription was done at 42°C for 2 hours; the enzyme was inactivated at 68°C for 10 minutes, and the sample was then held at 4°C until processed further. Two microliters of the RT reaction was used for each PCR reaction. The PCR process was performed by use of 1.25 units of *Taq* DNA polymerase<sup>g</sup> and canine gene specific primers corresponding to collagen type II, aggrecan, **metalloproteinase (MMP)-1**, **MMP-13**, **tissue inhibitor of metalloproteinase (TIMP)-1**, **TIMP-2**, and **glyceraldehydes-3-phosphate dehydrogenase (GAPDH)**; Appendix). The RT-PCR profile consisted of an initial incubation at 94°C (5 minutes), followed by 40 cycles of 15 seconds at 94°C, 30 seconds at 55°C, 30 seconds at 72°C, and a final extension step of 7 minutes at 72°C. The RT-PCR products were visualized on a 2% agarose gel containing ethidium bromide (0.5 µg/mL). To quantify the intensity of ethidium bromide signals, the gels were photographed by use of an electrophoresis documentation and analysis system<sup>h</sup> and the images were analyzed with 1-D image analysis software.<sup>h</sup> The net intensity of each gene's RT-PCR product was determined and compared with the net intensity of the corresponding GAPDH RT-PCR product to determine relative levels of gene expression. Results are expressed as the ratio of net intensity of a sample to net intensity of the GAPDH RT-PCR product. The no-RT controls were negative for each primer set used, indicating that genomic DNA contamination was undetectable.

**Inflammatory mediators in conditioned media**—Concentrations of PGE<sub>2</sub> in samples of conditioned media were quantitated via enzyme immunoassay according to protocols of the manufacturer.<sup>i</sup> In addition, because nitrite is the stable end product of nitric oxide oxidation, the concentrations of nitrite in samples of media were quantitated as an indicator of nitric oxide synthesis. Nitrite concentration in a sample of medium was measured spectrophotometrically by use of the Griess reaction with sodium nitrite as the standard.<sup>17</sup> An aliquot of 50 µL of collected culture medium was incubated with the same volume of 0.1% sulfanilamide in 5% phosphoric acid and with the same volume of 0.1% *N*-1-naphthyl-ethylenediamine dihydrochloro-

ride<sup>j</sup> for 10 minutes before measurement of absorbance at 550 nm.

**Statistical analyses**—Statistical analyses were performed by use of a computer software program.<sup>k</sup> Data from each collection day in each group were combined, and mean values  $\pm$  SEM were determined. A 1-way ANOVA was performed to determine differences among treatment groups with respect to each assay at each collection time. When significant differences among groups were obtained, multiple comparisons (Dunnnett method) were performed to determine which treatment groups were different from the control group. Significance was established at a value of  $P < 0.05$ .

## Results

**Extracellular matrix concentrations in 3-D constructs**—As determined via the DMMB assay, GAG concentrations in the control group 3-D constructs were significantly ( $P < 0.05$ ) higher than findings in all other treatment groups at day 12 (Figure 1). No other significant differences were detected among groups at each sample collection time. The GAG concentrations in samples of conditioned media were not significantly different among groups at each sample collection time (data not shown).

**Semiquantitative RT-PCR assay**—Compared with findings in the control groups, the relative gene expression level of type II collagen was significantly ( $P < 0.05$ ) decreased on day 1 in the IL-1 $\beta$ (20) and IL-1 $\beta$ (100) treatment groups and on day 3 in the IL-1 $\beta$ (20) treatment groups (Figure 2). Throughout the study period, there were no significant differences between controls and each IL-1 $\beta$  group with respect to the expressions of aggrecan and MMP-1 (Figures 2 and 3). In the IL-1 $\beta$ (50) treatment group, the relative expression levels of MMP-13, TIMP-1, and TIMP-2 on day 1 were significantly ( $P < 0.05$ ) increased, compared with findings in the control groups. Compared with control group data, the relative expression levels of MMP-13 in the IL-1 $\alpha$ (20) treatment group on days 3 and 6 were significantly ( $P < 0.05$ ) increased and the relative expression level of TIMP-2 in the IL-1 $\alpha$ (50) treatment group on day 12 was significantly ( $P < 0.05$ ) decreased. Type II collagen expression was significantly ( $P < 0.05$ ) decreased in the TNF- $\alpha$ (50) treatment group on day 6, whereas aggrecan expression was significantly ( $P < 0.05$ ) decreased in the TNF- $\alpha$ (20) and TNF- $\alpha$ (50) treatment groups on

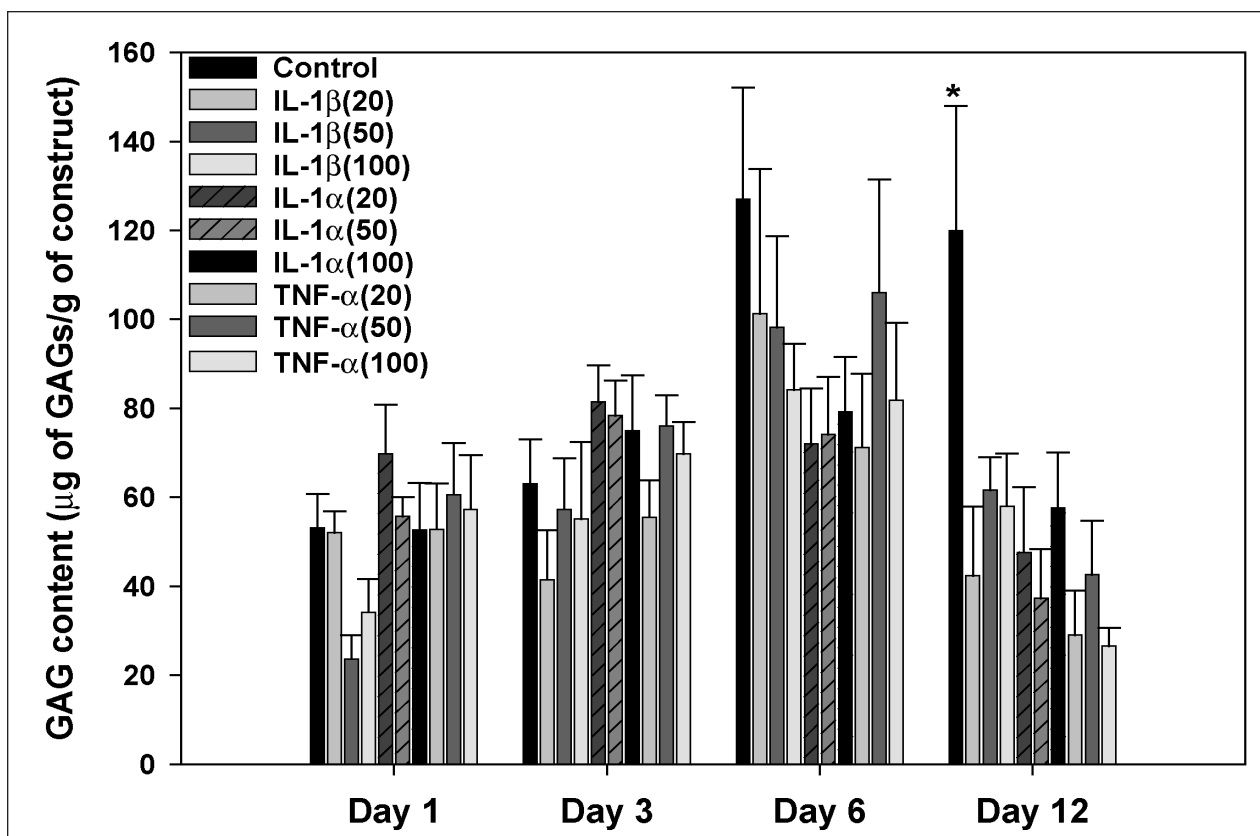


Figure 1—Glycosaminoglycan (GAG) content (determined via dimethylmethylene blue assay) of the extracellular matrix in 3-dimensional (3-D) constructs of chondrocytes isolated from humeral head articular cartilage obtained from 6 adult dogs after culture with interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , or tumor necrosis factor (TNF)- $\alpha$  at concentrations of 20, 50, or 100 ng/mL for 1, 3, 6, or 12 days. Treatments were applied as follows: control group, 3-D constructs in conditioned medium alone; IL-1 $\beta$ (20) group, 3-D constructs in conditioned medium with IL-1 $\beta$  (20 ng/mL); IL-1 $\beta$ (50) group, 3-D constructs in conditioned medium with IL-1 $\beta$  (50 ng/mL); IL-1 $\beta$ (100) group, 3-D constructs in conditioned medium with IL-1 $\beta$  (100 ng/mL); IL-1 $\alpha$ (20) group, 3-D constructs in conditioned medium with IL-1 $\alpha$  (20 ng/mL); IL-1 $\alpha$ (50) group, 3-D constructs in conditioned medium with IL-1 $\alpha$  (50 ng/mL); IL-1 $\alpha$ (100) group, 3-D constructs in conditioned medium with IL-1 $\alpha$  (100 ng/mL); TNF- $\alpha$ (20) group, 3-D constructs in conditioned medium with TNF- $\alpha$  (20 ng/mL); TNF- $\alpha$ (50) group, 3-D constructs in conditioned medium with TNF- $\alpha$  (50 ng/mL); and TNF- $\alpha$ (100) group, 3-D constructs in conditioned medium with TNF- $\alpha$  (100 ng/mL). Data are presented as mean values  $\pm$  SEM. \*On day 12 of culture, GAG content in the control group was significantly ( $P < 0.05$ ) higher than that of all other treatment groups. No other significant differences among groups at each sample collection time were detected.

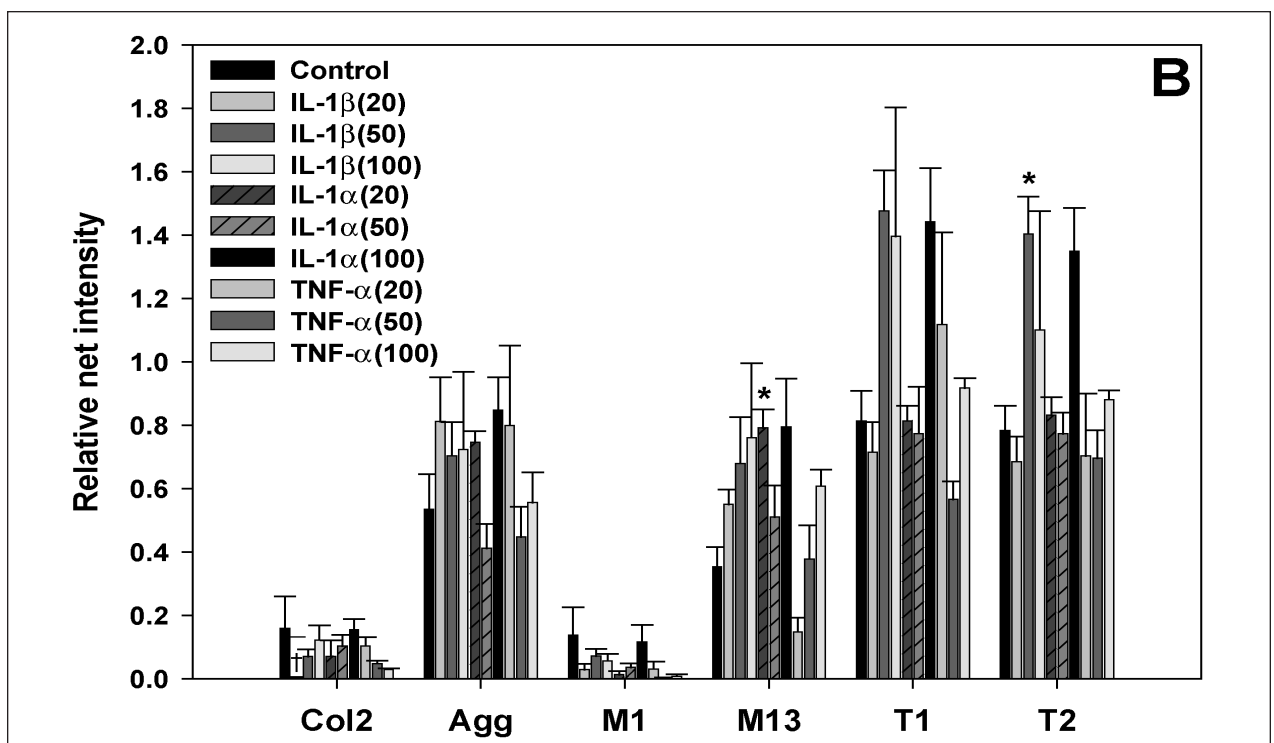
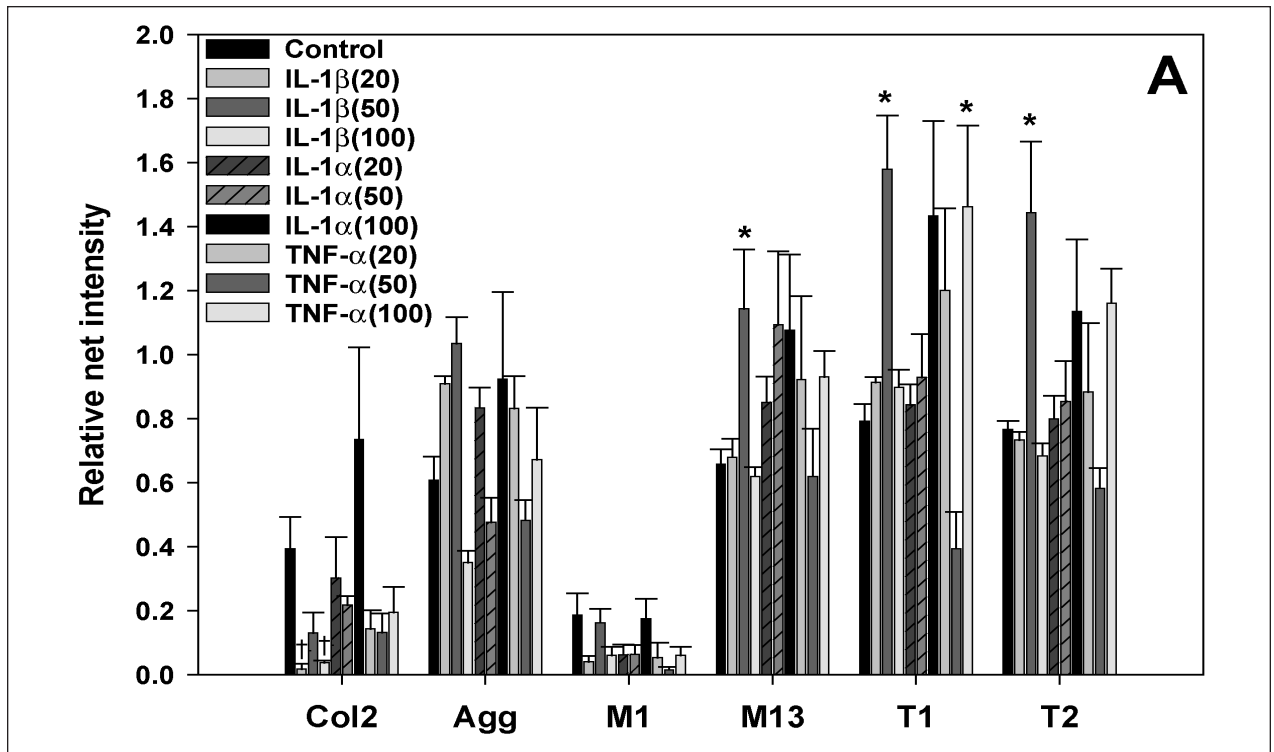


Figure 2—Ratio of the net intensity of the reverse-transcription polymerase chain reaction (RT-PCR) product for type II collagen (Col2), aggrecan (Agg), metalloproteinase (MMP)-1 (M1), MMP-13 (M13), tissue inhibitor of metalloproteinase (TIMP)-1 (T1), and TIMP-2 (T2) to the net intensity of the corresponding glyceraldehydes-3-phosphate dehydrogenase (GAPDH) RT-PCR product (relative net intensity) in samples of media from cultures of canine chondrocytes after exposure to IL-1 $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  at concentrations of 20, 50, or 100 ng/mL. A—Results after 1 day of culture. Compared with findings in the control groups, gene expressions of MMP-13, TIMP-1, and TIMP-2 in the IL-1 $\beta$ (50) group and TIMP-1 in the TNF- $\alpha$ (100) group were significantly ( $P < 0.05$ ) increased, whereas type II collagen expression in the IL-1 $\beta$ (20) and IL-1 $\beta$ (100) groups were significantly ( $P < 0.05$ ) decreased. B—Results after 3 days of culture. Compared with findings in the control groups, TIMP-2 expression in the IL-1 $\beta$ (50) group was significantly increased; in the IL-1 $\beta$ (20) group, type II collagen expression was significantly decreased and in the IL-1 $\alpha$ (20) group, MMP-13 gene expression was significantly increased. \*In each panel, value was significantly ( $P < 0.05$ ) increased, compared with that of the control group within that assessment category. †In all panels, value was significantly ( $P < 0.05$ ) decreased, compared with that of the control group within that assessment category. See Figure 1 for key.

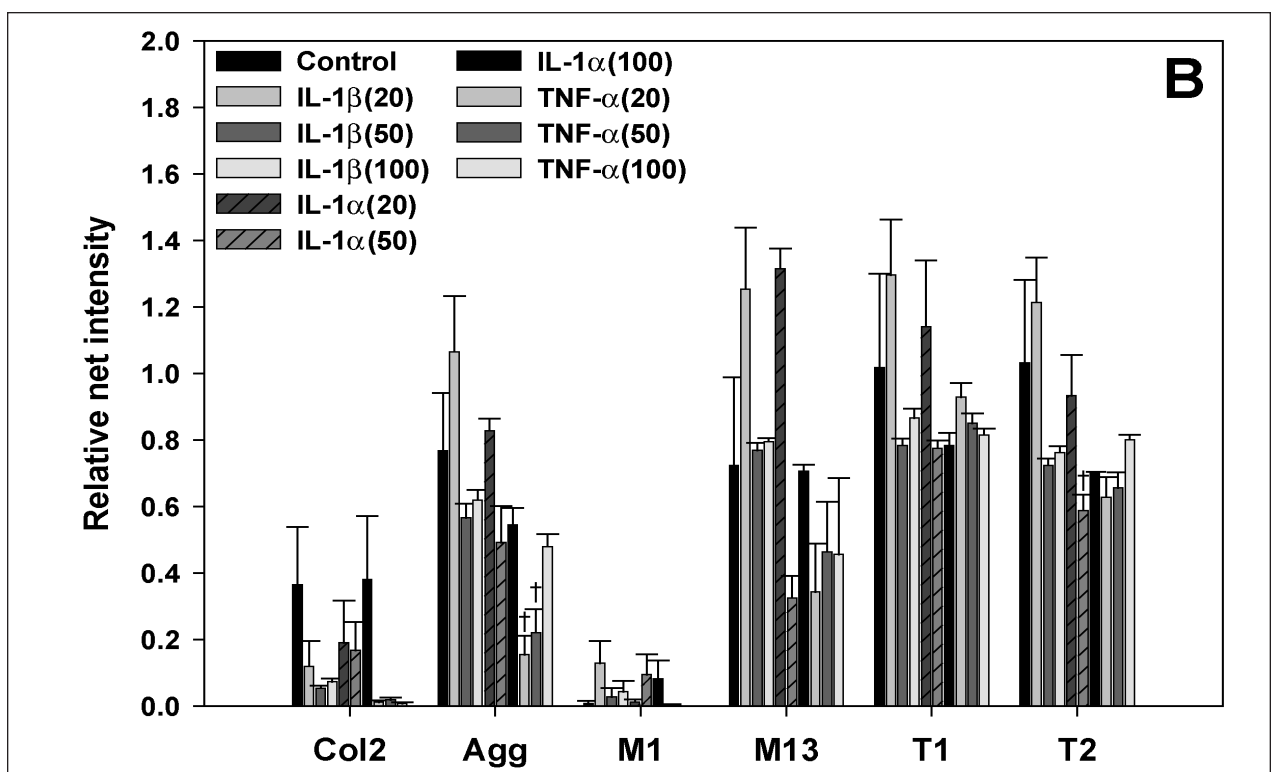
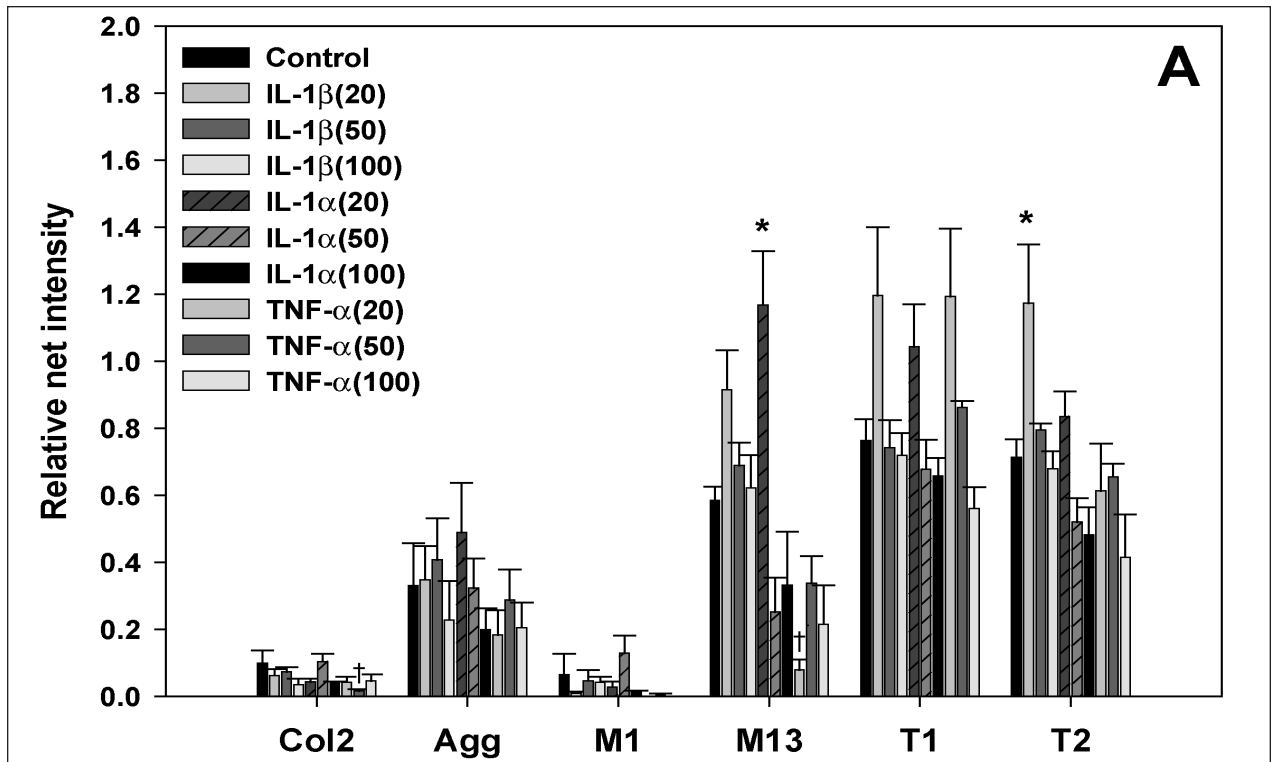


Figure 3—Ratio of the net intensity of the RT-PCR product for type II collagen (Col2), aggrecan (Agg), MMP-1 (M1), MMP-13 (M13), TIMP-1 (T1), and TIMP-2 (T2) to the net intensity of the corresponding GAPDH RT-PCR product (relative net intensity) in samples of media from cultures of canine chondrocytes after exposure to IL-1 $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  at concentrations of 20, 50, or 100 ng/mL. A—Results after 6 days of culture. Compared with findings in the control groups, MMP-13 expression in the IL-1 $\alpha$ (20) group was significantly increased; MMP-13 gene expression in the TNF- $\alpha$ (20) group and type II collagen expression in the TNF- $\alpha$ (50) group were significantly decreased. B—Results after 12 days of culture. Compared with findings in the control group, aggrecan gene expressions in the TNF- $\alpha$ (20) and TNF- $\alpha$ (50) groups and TIMP-2 gene expression in the IL-1 $\alpha$ (50) group were significantly decreased. \*In each panel, value was significantly ( $P < 0.05$ ) increased, compared with that of the control group within that assessment category. In all panels, value was significantly ( $P < 0.05$ ) decreased, compared with that of the control group within that assessment category. See Figure 1 for key.

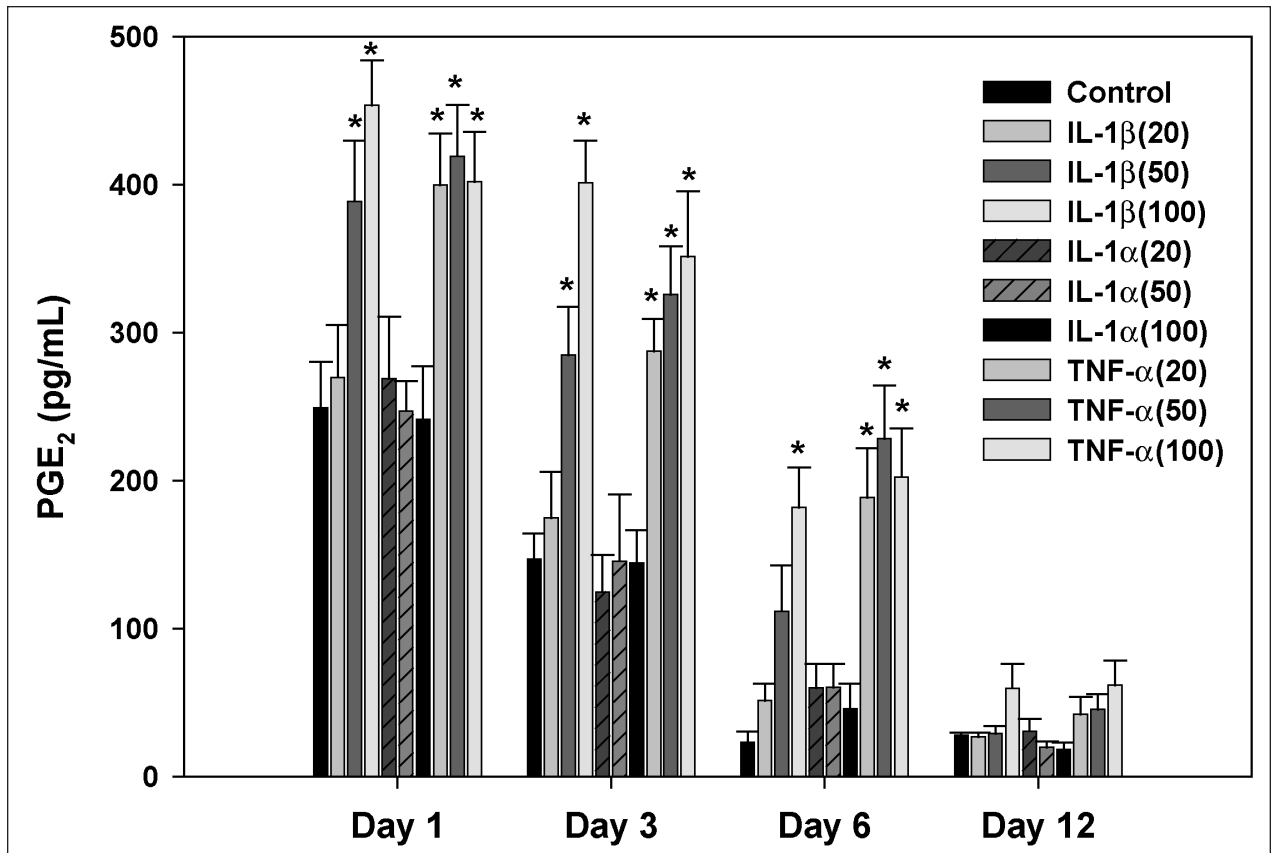


Figure 4—Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentrations (determined via an enzyme immunoassay) in samples of media from cultures of canine chondrocytes after exposure to IL-1 $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  at concentrations of 20, 50, or 100 ng/mL for 1, 3, 6, or 12 days. The PGE<sub>2</sub> concentrations in samples of culture media from the IL-1 $\beta$  treatment groups increased in a dose-dependent manner. Compared with findings in the control groups, PGE<sub>2</sub> concentrations in samples from the IL-1 $\beta$ (50) and IL-1 $\beta$ (100) groups after 1 and 3 days of culture and in the IL-1 $\beta$ (100) group after 6 days of culture were significantly increased; PGE<sub>2</sub> concentrations in samples from all TNF- $\alpha$  treatment groups were also significantly increased after 1, 3, and 6 days of culture. In media samples from the IL-1 $\alpha$  treatment groups, PGE<sub>2</sub> concentrations did not vary significantly from control group values. \*Value was significantly ( $P < 0.01$ ) increased, compared with that of the control group within that time point. See Figure 1 for key.

day 12, compared with findings in the control groups. The relative expression level of TIMP-1 was significantly ( $P < 0.05$ ) higher in the TNF- $\alpha$ (100) treatment group on day 1, compared with control group data. There were no other significant differences among groups at any of the sample collection times.

**Inflammatory mediators in conditioned media**—Compared with findings in the control groups, PGE<sub>2</sub> concentrations in samples of conditioned media from the IL-1 $\beta$  groups were increased in a dose-dependent manner; concentrations in the IL-1 $\beta$ (50) and IL-1 $\beta$ (100) groups were significantly higher ( $P < 0.01$ ) than concentrations in samples of conditioned media from the control groups at days 1 and 3 (Figure 4). The concentration of PGE<sub>2</sub> in the sample of medium from the IL-1 $\beta$ (100) group was also significantly ( $P < 0.01$ ) higher at day 6 than that detected in the control group sample. In samples of media from the TNF- $\alpha$  treatment groups, PGE<sub>2</sub> concentration was increased regardless of the concentration of TNF- $\alpha$ , compared with that detected in control group samples; PGE<sub>2</sub> concentrations in samples of media from the TNF- $\alpha$  treat-

ment groups were significantly ( $P < 0.01$ ) higher than control group values throughout the study period, except at day 12. Throughout the study period, PGE<sub>2</sub> concentrations in samples of media were not significantly affected in any of the IL-1 $\alpha$  treatment groups, compared with findings in control group samples.

Nitric oxide concentrations (determined as nitrite concentrations) in samples of conditioned media were increased only in TNF- $\alpha$  groups, compared with findings in control group samples (Figure 5); nitric oxide concentration in the samples of media from TNF- $\alpha$  groups were significantly ( $P < 0.001$ ) increased throughout the study period regardless of the concentration of TNF- $\alpha$ . Throughout the study period, nitric oxide concentrations in samples of media were not significantly affected in any of the IL-1 $\beta$  and IL-1 $\alpha$  treatment groups, compared with findings in control group samples.

## Discussion

Three-dimensional agarose gel culture systems have been widely used for investigating the characteristics of articular chondrocytes in vitro because such systems allow maintenance of native chondrocyte phe-

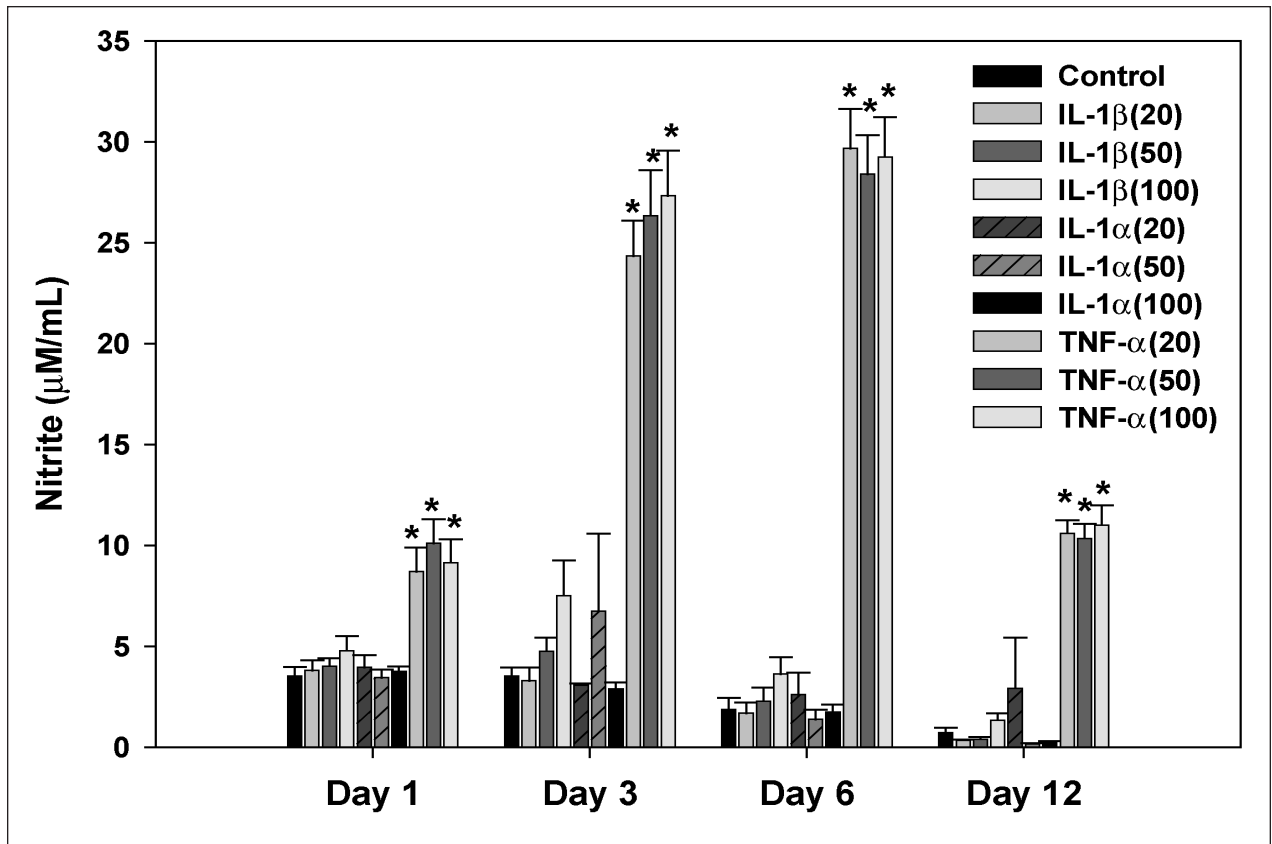


Figure 5—Nitric oxide concentrations (determined as nitrite concentrations via Griess assay) in samples of media from cultures of canine chondrocytes after exposure to IL-1 $\alpha$ , IL-1 $\beta$ , or TNF- $\alpha$  at concentrations of 20, 50, or 100 ng/mL for 1, 3, 6, or 12 days. Throughout the study period, nitric oxide concentrations in samples of media from all TNF- $\alpha$  treatment groups were significantly increased, compared with findings in the control groups. In media samples from the IL-1 $\alpha$  and IL-1 $\beta$  treatment groups, nitric oxide concentrations did not vary significantly from control group values. \*Value was significantly ( $P < 0.001$ ) increased, compared with that of the control group within that time point. See Figure 1 for key.

notype in culture and enable induction of inflammation and degradation of cell cultures by use of recombinant human cytokines.<sup>13-15,18</sup> After initial culture in a monolayer, reestablishment of chondrocyte phenotype occurs within the first 2 weeks of 3-D culture<sup>18</sup>; therefore, in the present study, chondrocytes were cultured for 2 weeks in the 3-D system prior to exposure to cytokines to minimize the influences of such chondrocyte redifferentiation on the assessments of treatment effects. In addition, serum in the conditioned culture media was withdrawn during the study period to avoid confounding effects of serum proteins, which might exaggerate or diminish the production of matrix molecules and catabolic factors targeted in the present study. The low cellularity and dense extracellular matrix of articular cartilage make the extraction of considerable quantities of high-quality mRNA difficult. A 3-D culture system can be a feasible way to perform a systematic investigation of chondrocyte gene expression under various stimuli because the cellularity can be controlled to facilitate higher extraction yields of RNA.

In the present study, the effect of proinflammatory cytokines on the relative gene expression levels for the genes investigated was inconclusive due in part to a lack of observable dose-dependent changes in any of the treatment groups. However, some general

characterizations of the experimental system could be determined from these data. For example, the relative expression levels of TIMP-1 and TIMP-2 were consistently high, whereas the relative expression level of MMP-1 was consistently low. The large variability in the relative gene expression levels observed in our study may be attributed to the methods used. First, it is possible that the quality of RNA obtained from agarose constructs was relatively poor or inconsistent, resulting in large differences in relative expression levels among samples. It has been observed in our laboratory that RNA extraction from agarose gels results in lower RNA yield, compared with yields of RNA extracted from monolayer culture and large tissue samples. Second, preliminary data for the present study indicated that 40 cycles of the RT-PCR assay were required to ensure detection in all samples. It is probable that the level of expression was determined after the RT-PCR assay was past the exponential phase in some samples, resulting in the underestimation of the level of expression for that sample. In our laboratory, we now use newer real-time RT-PCR protocols that more precisely measure the relative expression levels for each gene; the use of such protocols may decrease the variability of gene expression results obtained in this type of research. Furthermore, the viability of 3-D cultured chondro-

cytes was not evaluated in the present study. It is possible that the lack of dose-dependent changes in relative gene expression levels was a result of decreased cell viability in cultures exposed to higher concentrations of the proinflammatory cytokines.

Compared with control group data, GAG content in 3-D constructs in all treatment groups was significantly decreased at day 12, which indicates that each recombinant human cytokine at concentrations of 20 ng/mL or more can cause unfavorable effects on the proteoglycan matrix. The decreased GAG content can be attributed to decreased synthesis, increased loss of proteoglycans, or both. Although gene expression levels are not always reflective of protein synthesis, the fact that aggrecan gene expression levels remained unchanged in all treatment groups throughout the study period (with the exception of levels in 2 TNF- $\alpha$  groups at day 12), compared with aggrecan gene expression levels in the control groups, suggests that the decreased GAG content associated with cytokine treatments in the present study does not solely stem from decreased synthesis. In addition, lack of significant increases in GAG content in samples of conditioned media from the treatment groups suggests that increased degradation is also not primarily responsible for decreased GAG content in the 3-D constructs. However, it is possible that the GAG quantification method used in our study was not sensitive enough to accurately measure GAG concentrations in samples of culture media, and other methods may be necessary to fully assess proteoglycan degradation. Recently, the role of MMPs in proteoglycan degradation has been overshadowed by that of aggrecanases, which cleave in the interglobular domain of aggrecan that is present in Glu<sup>373</sup>-Ala<sup>374</sup> bonds.<sup>19,20</sup> Although MMPs, including the collagenases, are reported to be capable of cleaving aggrecan in the interglobular domain in Asn<sup>373</sup>-Phe<sup>374</sup> bonds,<sup>21</sup> results of *in vitro* studies by Little et al<sup>19</sup> and Tortorella et al<sup>20</sup> indicated that aggrecan degradation following proinflammatory cytokine exposure of cartilage explants was primarily the result of aggrecanase activity and not MMP activity. The use of a sulfate incorporation assay involving radioactive sulfur (<sup>35</sup>S) or an immunoassay to detect aggrecanase-derived proteoglycan neoepitopes<sup>19,20</sup> would aid in investigations to determine the mode of proteoglycan changes that account for the decreased GAG content in 3-D constructs detected in our study.

Compared with control group values, significant increases in the relative gene expression levels of MMP-13, TIMP-1, and TIMP-2 in the IL-1 $\beta$ (50) group at day 1 and TIMP-2 in IL-1 $\beta$ (20) at day 6 imply a dual role for IL-1 $\beta$  in our experimental system. The induction of MMP-13 expression, which has been reported to cleave type II collagen at least 10-fold faster than MMP-1,<sup>22</sup> suggests a role for IL-1 $\beta$  in matrix degradation. Conversely, increases in the relative expression levels of TIMP-1 and TIMP-2 (which inactivate MMPs by binding to MMPs at a 1:1 [molar ratio] stoichiometric ratio) indicate a matrix-protective role for IL-1 $\beta$ . Excessive amounts of MMPs, compared with amounts of TIMPs, in joint tissues from humans with osteoarthritis have

been reported.<sup>23,24</sup> It can be speculated that IL-1 $\beta$  activity *in vivo* results in changes in both MMP and TIMP gene expression levels to at least some degree during development of osteoarthritis and likely does not foster the maintenance of the precise balance between MMP and TIMP concentrations.

It is noteworthy that recombinant human IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$  regulate PGE<sub>2</sub> and nitric oxide production by canine chondrocytes differently. Both PGE<sub>2</sub> and nitric oxide are potent mediators of chondrocyte metabolism. Excess production of nitric oxide has been linked with inhibition of chondrocyte proliferation, decreased matrix synthesis, and induction of apoptosis.<sup>25-28</sup> In the present study, it is possible that increases in nitric oxide concentrations caused apoptosis of chondrocytes and affected the extracellular matrix composition of the cultures. Further investigations to determine cell viability in cultures and assess the effects of nitric oxide inhibitors in the experimental system used in our study are warranted to further delineate the roles of nitric oxide in relationship to TNF- $\alpha$  activity and cellular and extracellular matrix changes of chondrocytes. The dose-dependent increases in the production of PGE<sub>2</sub> in 3-D-cultured chondrocytes exposed to recombinant human IL-1 $\beta$  detected in the present study are in agreement with findings of a previous study<sup>13</sup> performed by our group. Results of the present study clearly indicate that production of PGE<sub>2</sub> in cultured canine chondrocytes can be significantly stimulated by TNF- $\alpha$  at a concentration of at least 20 ng/mL.

It has been reported that compressive loads alter PGE<sub>2</sub> synthesis by chondrocytes through a nitric oxide-dependent pathway.<sup>29,30</sup> Results of previous studies<sup>30,31</sup> have indicated interactions between chondrocyte regulation of PGE<sub>2</sub> and nitric oxide in that PGE<sub>2</sub> production was enhanced through the suppression of nitric oxide synthesis. Our analyses of samples of culture media revealed that there was a concomitant increase in PGE<sub>2</sub> and nitric oxide concentration in the TNF- $\alpha$  treatment groups and an increase in PGE<sub>2</sub> concentration without a change in nitric oxide concentration in the IL-1 $\beta$  treatment groups, which lead us to hypothesize that recombinant human TNF- $\alpha$  may regulate nitric oxide and PGE<sub>2</sub> production by canine chondrocytes through interconnected pathways, whereas recombinant human IL-1 $\beta$  may stimulate canine chondrocytes to produce PGE<sub>2</sub> through a nitric oxide-independent pathway. In the present study, the decreases in PGE<sub>2</sub> concentrations in each treatment group during the study period might stem from alterations in cell metabolism or cell viability in response to altered cell nutrition (ie, the provision of serum-free medium).

Increased synthesis of IL-1 receptor type I (IL-1RI) associated with enhanced MMP production has been identified in articular chondrocytes collected from osteoarthritic joints of humans.<sup>32</sup> Interaction between IL-1RI and an IL-1RI accessory protein drives the IL-1 signaling pathway.<sup>33,34</sup> It is uncertain whether canine chondrocytes have different affinities for human recombinant IL-1 $\alpha$  and IL-1 $\beta$ . On the basis of results of our study, the hypothesis that the effects of those 2 recombi-



nant human proinflammatory cytokines on 3-D cultured canine chondrocytes would be significantly different was accepted. However, it is apparent that recombinant human IL-1 $\alpha$  is not an optimal choice for use in vitro in canine chondrocyte-based osteoarthritis experiments, especially those in which the inflammatory aspects of the disease process are investigated. For in vitro osteoarthritis experiments involving canine chondrocytes, it is important to consider the use of IL-1 $\beta$  with TNF- $\alpha$  and attempt to achieve optimal concentrations and concentration ratios of IL-1 $\beta$  to TNF- $\alpha$  to induce cartilage degradation similar to that detected in joints with naturally occurring osteoarthritis. It is likely that these and other proinflammatory cytokines and inflammatory mediators interact intricately in the disease progression. Although identification of a sole etiologic factor for development of osteoarthritis in humans and other animals is unlikely, studies that focus on the investigation of cellular characteristics of articular chondrocytes may deepen our understanding of the disease and provide vital information for the prevention and efficacious treatment of osteoarthritis.

- a. Fisher Scientific, Fair Lawn, NJ.
- b. R&D Systems Inc, Minneapolis, Minn.
- c. Sigma Chemical Co, St Louis, Mo.
- d. RNeasy mini-kit, Qiagen, Valencia, Calif.
- e. Integrated DNA Technologies, Skokie, Ill.
- f. Stratagene, La Jolla, Calif.
- g. Invitrogen, Carlsbad, Calif.
- h. KODAK electrophoresis documentation and analysis system (EDAS) 120, Eastman Kodak Co, Rochester, NY.
- i. Amersham Biosciences, Piscataway, NJ.
- j. Promega, Madison, Wis.
- k. SigmaStat, version 2.03, Jandel Scientific, San Rafael, Calif.

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## Appendix

Oligonucleotide primers used for reverse-transcription polymerase chain reaction assays performed on agarose-based 3-dimensional constructs of cultured chondrocytes isolated from humeral head articular cartilage obtained from 6 adult dogs. Primer sequences correspond to cDNA sequences deposited in GenBank.

Target template	Primers	Amplification product size (bp)
Type II collagen	F5'-TGGAGCAGCAAGAGCAAGGA-3' RC5'-ATCAGGTCAGGTCAGCCATTC-3'	563
Aggrecan	F5'-ACTGTTTCCCAGGAACCTTGC-3' RC5'-TGGGTCTCCTCTCCTGAGT-3'	607
Metalloproteinase-1	F5'-TACGGATACCCCAAGGACAT-3' RC5'-CTGCAGTTGAACCAGCTATT-3'	328
Metalloproteinase-13	F5'-GCCTTCCTCTTCTTGAGCTG-3' RC5'-TTGGACCACTTGAGAGTTCG-3'	320
Tissue inhibitor of metalloproteinase-1	F5'-GCCCAGAGAGACTCACCAGA-3' RC5'-CTCACAGCCAGCAGCATAGG-3'	472
Tissue inhibitor of metalloproteinase-2	F5'-GACCAGACAAGGACATAGAG-3' RC5'-TCAGAGCTGGACCAGTCTAA-3'	488
Glyceraldehydes- 3-phosphate dehydrogenase	F5'-CCACGGCAAATCCACGGGCACAG-3' RC5'-GGGGTCCCTCCGATGCCTGCTTC-3'	652