The effects of TIMP-1 and -2 on canine chondrocytes cultured in three-dimensional agarose culture system

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Summary

Objective: To investigate the effects of tissue inhibitor of metalloproteinase (TIMP)-1 and -2 on chondrocytes cultured with or without interleukin (IL)-1β.

Design: Canine articular chondrocytes were cultured in three-dimensional (3-D) agarose constructs. Cells were distributed into each of the two groups, those without IL-1β and those with IL-1β added to the liquid media. Each group was subdivided into three groups, based on the presence of TIMP-1 or -2. IL-1β and TIMPs were added to liquid media bathing the 3-D constructs beginning on day 3. The liquid media and the 3-D constructs were collected on days 9, 15, and 24, and analyzed histologically, biochemically, and immunohistochemically.

Results: Addition of TIMP-1 or -2 resulted in decreases in matrix metalloproteinase (MMP)-3 concentrations of 37 and 41%, and MMP-1 concentrations of 28 and 34% in 3-D constructs of groups without IL-1β and with IL-1β, respectively, on day 9. Chondrocytes in groups without IL-1β maintained viability and produced abundant extracellular matrix (ECM). Chondrocytes in IL-1β groups appeared less viable and produced less ECM compared with those without IL-1β.

Conclusions: The addition of TIMP was not detrimental to chondrocytes, as used in this study. Despite evidence of decreased MMP levels, TIMPs did not prevent IL-1β-associated changes in cellular or ECM characteristics. Further study is necessary before clinically relevant conclusions can be drawn regarding the use of TIMPs in the treatment of osteoarthritis.

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Key words: Chondrocytes, Tissue inhibitor of metalloproteinases, Osteoarthritis, Interleukin-1β.

Introduction

Tissue inhibitors of matrix metalloproteinases (TIMPs) are known endogenous protease inhibitors that bind to active matrix metalloproteinases (MMPs) in a 1:1 molar ratio stoichiometry1,2. Although the pathogenesis of osteoarthritis (OA) is not fully understood, MMPs derived from chondrocytes, synovium, and polymorphonuclear leukocytes have been proposed to play a major role in cartilage degradation seen in OA3,4. The balance between MMPs and TIMPs is tightly controlled in healthy joints. However, in OA, the amount of MMPs exceeds the locally available TIMPs, resulting in excessive extracellular matrix (ECM) degradation5,6. Hence, MMPs have been considered as potential targets for therapeutic strategies for OA. Therapeutic strategies for MMP regulation can include control of synthesis and secretion of MMPs, inhibition of conversion of the latent form, proMMPs, to active form MMPs, and direct inhibition of active MMPs7,8. TIMPs provide a potential therapeutic modality for decreasing the detrimental effects of MMPs through direct inhibition.

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Inhibition of metalloproteinase activities has been reported in chondrocyte culture9-10, cartilage explant culture11-15, and animal studies using MMP inhibitors, including tetracycline16, chemically derived MMP inhibitors (1,10-phenanthroline, EDTA, razoxane, and 6,7-dihydroxycoumarin)9-11, bacterially derived MMP inhibitor (acxinonin)17, and physiological MMP inhibitors (TIMP-1 and -2)9,12,15. Hughes et al.9 reported that more than 90% of MMP activity in conditioned media from porcine chondrocytes cultured in the presence of interleukin (IL)-1α was inhibited by incubation with human recombinant TIMP-1 (255 nM) or TIMP-2 (285 nM). Bonassar et al.12 reported that addition of 4 µM TIMP-1 to the culture media of bovine cartilage explants inhibited IL-1β-induced loss of tissue sulfated glycosaminoglycan (GAG) by 40% after 8 days in culture. Ellis et al.15 reported that both TIMP-1 and -2 completely prevented the release of collagen fragments from bovine nasal cartilage exposed to IL-1α. MMP-induced degradation and release of GAG and collagen fibrils from articular cartilage are hallmarks of OA. The lack of GAG comprising proteoglycan aggrecan in osteoarthritic articular cartilage exacerbates insufficient compressive stiffness properties, and the damage to collagen fibrils results in less effective tensile strength of the tissues, leading to further mechanical disruption of the cartilage even under physiological loads. Developing therapeutic interventions that alter the progression of OA is critical, and the use of TIMPs would appear to be a potentially effective strategy.
However, to the authors’ knowledge, data are lacking in the literature with regard to the long-term effects of exogenous TIMPs on cellular and extracellular characteristics of chondrocytes. The present study was designed to elucidate the temporal effects of TIMP-1 and -2 on chondrocytes cultured with or without IL-1β, over a 3-week period, using a three-dimensional (3-D) agarose gel culture system.

Materials and methods

COLLECTION OF SAMPLES

Full-thickness articular cartilage slices (n=5) were aseptically obtained from the caudal central portion of the humeral head of five canine cadavers (age range, 12–24 months) via arthrotomy performed immediately after euthanasia. The dogs were euthanatized for reasons unrelated to this study, and were apparently healthy with grossly normal humeral head cartilage.

CHONDOCYTE CULTURE

Canine chondrocytes were isolated and cultured in monolayer for amplifying cell number prior to placement in 3-D agarose gel, as previously reported. At near confluency in monolayer culture, media (RPMI 1640 containing penicillin (100 IU/ml)/streptomycin (100 µg/ml)/amphotericin B (2.5 µg/ml) plus 10% fetal bovine serum (RPMI-FBS)) were discarded, the flasks rinsed, and the cells detached by trypsinization, washed in Hank's balanced salt solution (HBSS), resuspended in RPMI-FBS, and counted and checked for viability by trypan blue exclusion. Greater than 95% cell viability was ascertained in each sample. Equal volumes of 2% low-melting agarose (Fisher Scientific, Fair Lawn, NJ) (gelling temperature 25±5°C) in phosphate buffered saline and double-strength containing penicillin (100 IU/ml)/streptomycin (100 µg/ml)/amphotericin B (2.5 µg/ml) plus 10% fetal bovine serum (RPMI-FBS)) were added to produce a cell concentration of 10⁶ cells/ml. One milliliter of the cell suspension was added to the wells of a 24-well tissue culture plate. The plates were placed in a refrigerator at 4°C for 5 min. After the agarose medium was gelled to form the 3-D construct, 1 ml of RPMI-FBS liquid media was added to each well. The plates were incubated at 37°C with 5% CO₂ and 95% humidity. Recombinant human TIMP-1 (255 nM/ml; T1; Calbiochem, San Diego, CA), TIMP-2 (285 nM/ml; T2; Calbiochem, San Diego, CA), IL-1β (20 ng/ml; Upstate Biotechnology, Lake Placid, NY), IL-1β plus TIMP-1 (IL/T1), or IL-1β plus TIMP-2 (IL/T2) were added to liquid medium bathing the 3-D constructs, beginning on day 3 of 3-D culture and refreshed every 3 days. In the present study, the samples were divided into two groups based on the presence or absence of IL-1β. For determination of the effects of TIMP-1 and -2, each group was subdivided into three groups based on the presence of TIMP-1 or -2. The chondrocytes cultured in media (RPMI-FBS), with no IL-1β or TIMP, served as controls.

SAMPLE COLLECTION

On days 9, 15, and 24 of 3-D culture, samples of liquid media were collected and stored at −80°C for subsequent evaluations for GAG and MMP-3 concentrations. The 3-D constructs in each well collected on days 9, 15, and 24 were divided into two portions. One portion was placed in 10% buffered formalin for histologic processing. The other portion was lyophilized, weighed, and stored at −20°C for subsequent evaluations for DNA, GAG, and hydroxyproline (HP) contents.

HISTOLOGIC EVALUATION

After routine histologic processing, 5 µm sections were stained with hematoxylin and eosin (H&E) and toluidine blue. Sections were subjectively evaluated in duplicate by two investigators (KK, JLC), who were unaware of collection day or group. Sections were evaluated for cellular characteristics of viability and morphology, and for ECM staining.

IMMUNOHISTOCHEMICAL EVALUATION

Unstained 5 µm sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by immersion in 3% H₂O₂ in methanol. After antigen retrieval treatment using trypsin for MMPs and type II collagen and boiled citrate buffer for type I collagen, non-specific binding was blocked with normal blocking serum. Slides were incubated overnight at 4°C for collagen type II (rabbit, anti-bovine) (Chemicon International Inc., Temecula, CA) (1:400 dilution), MMP-1 (mouse, anti-human) (Calbiochem, San Diego, CA) (1.0 µg/ml), MMP-13 (mouse, anti-human) (Calbiochem, San Diego, CA) (2.0 µg/ml), and at 37°C for collagen type I (goat, anti-human) (Chemicon International Inc., Temecula, CA) (1:150 dilution). The following day, the slides were incubated with biotinylated secondary antibody (DAKO, Carpinteria, CA). Bound primary antibody was detected using an avidin–horseradish peroxidase method with diaminobenzidine (DAB) chromogenic substrate (DAKO, Carpinteria, CA). Each antibody’s specificity for canine cartilage tissues has been verified by immunohistochemistry, as reported elsewhere. Each MMP antibody is reported to recognize latent and active forms without cross-reacting with other MMPs. Sections were counterstained with hematoxylin solution. Sections were subjectively examined by two investigators (KK, JLC), who were blinded as to the origin of each section evaluated. One hundred cells were counted on each slide, and the number of definitively positive-stained cells was recorded as percent-immunoreactivity.

MATRIX METALLOPROTEINASE-3 ASSAY

MMP-3 concentration in the liquid media was quantitated by enzyme linked immunosorbent assay (ELISA) (Calbiochem, San Diego, CA). The stored media were thawed and then assayed for MMP-3 content according to manufacturer’s instructions. As reported, this ELISA has been successfully utilized to determine MMP-3 concentrations in the liquid medium of cultured canine chondrocytes. The assay detects both latent and active MMP-3, but does not recognize MMP-3/TIMP complex. Sample concentrations determined from the calculated control curve were used for data analysis. MMP-3 concentration was reported in ng/ml.

GLYUCOSAMINOGLYCAN ASSAY

Total sulfated GAG content was quantitated by dimethyl-methylene blue (DMMB) assay. The 3-D samples were digested in 1 ml of 0.5 mg/ml papain (Sigma, St. Louis, MO) (14 U/mg) in 20 mM sodium phosphate buffer (pH 6.8) containing 1 mM EDTA at 60°C overnight. A 10 µl aliquot of
the digest solution was assayed for total GAG content by
addition of 240 µl of DMMB solution and by spectrophoto-
metric determination of absorbance at 525 nm. Known
concentrations of bovine trachea chondroitin sulfate A
(Sigma, St. Louis, MO) were used to construct the standard
curves. Total GAG content in 3-D samples was reported
in GAG/weight (µg/mg) and GAG/DNA (µg/µg). GAG
concentrations in the liquid media were reported in µg/ml.

DNA ASSAY

Aliquots of the papain digests prepared for DMMB assay
were utilized for DNA quantification, using a diphenylamine
assay24. A 10 µl aliquot of the digest solution was mixed
with acetaldehyde/perchloric acid followed by addition of
diphenylamine. Known concentrations of calf thymus DNA
(Sigma, St. Louis, MO) were used to construct the standard
curve. Absorbance of each sample was read at 595/700 nm
using a spectrophotometer.

HYDROXYPROLINE ASSAY

Total collagen content in 3-D constructs was determined
by measuring the HP content using a colorimetric pro-
cedure25. The papain digest solution prepared for DMBB
assay was utilized for HP assay. A 100 µl aliquot of the
digest solution was hydrolyzed with sodium hydroxide by
autoclaving. The hydrolyzate was mixed with chloramine
T reagent, followed by reaction with Ehrlich’s aldehyde
reagent. The sample was incubated at 65°C for 20 min to
develop a chromophore. Known concentrations of HP
(Sigma, St. Louis, MO) were used to construct the standard
curve. Absorbance of each sample was read at 550 nm
using a spectrophotometer. HP content in 3-D constructs
was reported in HP/weight (µg/mg).

STATISTICAL ANALYSIS

All assays were performed in duplicate. All statistical
analyses were performed using a computer software pro-
gram (Sigma Stat®, San Rafael, CA). Data from each
collection day in each group were combined, and means ±
standard errors (S.E.M.) were determined. A t-test
was performed to determine differences between groups
with IL-1β and without IL-1β, with respect to concentrations
of DNA, GAG, and HP at each sample collection time.
Significance was set at P<0.05. One-way ANOVA was
performed to determine differences among all treatment
groups with respect to each assay at each collection time.
When significant differences among groups were obtained,
an all-pairwise multiple comparison was performed to
determine which groups were different from each other.
Differences within a group with respect to each assay at
different collection times were analyzed in similar manner.

Results

HISTOLOGIC EVALUATION

It is known that TIMPs can have a direct inhibitory effect
on MMP activity. However, information regarding the effects
of TIMPs on chondrocytes is lacking. We, therefore, evalu-
ated cellular morphology to determine whether addition of
TIMP-1 or -2 had effects on chondrocytes cultured in the
presence or absence of IL-1β. Chondrocytes from all dogs
were successfully cultured in the 3-D agarose culture
system for the study duration. Chondrocytes in the control
appeared round to oval, with round basophilic nuclei at all
sample points. Chondrocytes in T1 and T2 groups showed
characteristics similar to that of the controls, as determined
by H&E-stained histomorphology (data not shown). Those
chondrocytes exposed to IL-1β (IL/T1, IL/T2) varied
in size, but a subjectively larger percentage of IL-insulted
chondrocytes were small cells with dense nuclei com-
pared with those cultured without IL-1β (controls, T1, T2).
Temporal matrix formation was examined by toluidine blue
staining. Purple-stained pericellular and territorial matrix
were evident in chondrocyte cultures without IL-1β, indicat-
ing the presence of abundant proteoglycan synthesis in
those groups [Fig. 1(A–C)]. Toluidine blue-stained matrix
in groups cultured without IL-1β increased in size and stain
intensity consistently throughout the study period. In

Fig. 1. Photomicrograph of a section of chondrocytes in 3-D culture from control (A), T1 (B), T2 (C), IL-1β (D), IL/T1 (E), and IL/T2 (F) on
day 15, isolated from same cartilage tissue demonstrating various cell and ECM differentiations among treatment groups. It is to be noted
that matrix formation and staining are more prominent in groups without IL-1β (A, B, C) compared with those with IL-1β (D, E, F).
Chondrocytes cultured with IL-1β showed wide variety of cellular characteristics within a section, from those chondrocytes that appear less
viable (pyknosis, karyorrhexis) (D), clustered chondrocytes and matrix with a lack of proteoglycan (E), to chondrocytes with stunted matrix
(F). Toluidine blue stain; bar=50 µm.
contrast, chondrocytes in groups with added IL-1β showed a variety of cell and matrix characteristics, including pyknotic chondrocytes, as shown in Fig. 1D, clustered chondrocytes with a lack of proteoglycan, as shown in Fig. 1E, and chondrocytes with small amounts of toluidine blue-stained matrix, as shown in Fig. 1F. The IL-1β group exhibited histologic characteristics of loss of cell viability, including nuclear pyknosis and lysis, more frequently than other groups. The histologic evaluations suggested that addition of TIMPs did not disturb cellular viability or matrix synthesis, nor did TIMPs prevent the detrimental effects of IL-1β on chondrocytes in this model.

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Decreased production, increased degradation, or both could be responsible for the inferior ECM formation noted in IL-insulted groups in this study. In addition, a study objective was to determine the effects of TIMPs on collagen synthesis. To address these questions, type I and II collagen immunohistochemistries were conducted using avidin–biotin–peroxidase staining method. Both type I and II collagens were evident in matrix by immunohistochemical assessment in each group at all sample collection times. A sparse number of chondrocytes were immunostained by anti-collagen type I, and there were no significant differences in the number of positive-stained chondrocytes among groups or within any group throughout the study period. When detected, positive type I collagen expression was noted in pericellular matrix, as shown in Fig. 2A. It has been reported that small amounts of type I collagen are present in 3-D cultures of chondrocytes, previously cultured in monolayer26,27. Therefore, it is likely that positive collagen type I immunostaining in this study resulted from phenotypic changes during monolayer culture and not from the effects of IL-1β or TIMPs.

Type II collagen was present in pericellular and territorial matrix of chondrocytes, as shown in Fig. 2B, in all groups. The number of positive-stained chondrocytes among groups or within any group throughout the study period. When detected, positive type II collagen expression was noted in pericellular matrix, as shown in Fig. 2A. It has been reported that small amounts of type I collagen are present in 3-D cultures of chondrocytes, previously cultured in monolayer26,27. Therefore, it is likely that positive collagen type I immunostaining in this study resulted from phenotypic changes during monolayer culture and not from the effects of IL-1β or TIMPs.

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(P=0.026) decreased in the IL/T2 group compared with the IL-1β group, and a similar trend was seen in IL/T1 group compared with the IL-1β group (P=0.066). MMP-1 immunoreactivities in IL/T1 and IL/T2 groups were 32 and 36% lower than those in the IL-1β group, respectively. These data suggest that addition of IL-1β induced increased MMP-1 production by chondrocytes, but did not influence MMP-13 levels as determined in the present study. Addition of TIMP-1 and -2 appeared to dampen IL-mediated increases in MMP-1 immunoreactivity. Relatively high numbers of MMP-1 positive chondrocytes in non-IL-1β exposure groups (control, T1, T2) may indicate a primary role for MMP-1 in matrix remodeling during redifferentiation and establishment of ECM by chondrocytes in 3-D agarose culture. Lower MMP-1 immunoreactivity in the IL-1β group later in 3-D culture may be related to a loss of cell viability and synthetic activity.

**MATRIX METALLOPROTEINASE ENZYME LINKED IMMUNOSORBENT ASSAY**

MMP-3 is known to degrade a wide spectrum of ECM macromolecules, including aggrecan. In an attempt to elucidate the histochemical findings in this study with respect to differences in amount and intensity of toluidine blue-stained matrix among groups, MMP-3 concentrations were determined in the liquid media using ELISA. MMP-3 concentrations in the liquid media were significantly (P=0.042) different among groups on day 9 (Fig. 6). The IL-1β group had significantly (P<0.05) higher MMP-3 concentrations compared with the groups not exposed to IL-1β (control, T1, T2) on day 9 (Fig. 6).
and -2 decreased MMP-3 levels compared with the IL-1β group by 37 and 41%, respectively. No other statistically significant differences among groups or within a group were noted. The results suggest that IL-1β treatment increased synthesis of MMP-3 early in 3-D culture. These data also suggest that exogenous TIMP-1 and -2 may bind to MMP-3 and suppress MMP-3 concentrations in the liquid media of 3-D chondrocyte cultures insulted with IL-1β.

**GLYCOSAMINOGLYCAN ASSAY**

To verify proteoglycan production as assessed by histochemical analysis, total GAG in the 3-D constructs was measured by DMMB assay. The results for GAG in 3-D normalized to construct weight (GAG/weight) corresponded well with the histochemical findings regarding toluidine blue-stained matrix formation. GAG in 3-D constructs increased significantly (P<0.001) during the study period within each group, except for groups IL-1β and IL/T2 [Fig. 7(A)]. There were significant (P<0.01) differences in GAG/weight among groups on days 15 and 24 [Fig. 7(A)].

![Fig. 7. Mean (±S.E.M.) GAG concentrations in 3-D constructs. Concentrations of GAG were measured on days 9, 15, and 24 of culture. (A) GAG in 3-D normalized by dry weights (GAG/weight) and (B) GAG in 3-D per DNA (GAG/DNA). GAG contents were significantly higher in non-IL-1β exposure groups (control, T1, T2) than those cultured with IL-1β (IL-1β, IL/T1, IL/T2) on days 15 and 24 in GAG/weight (A) and on day 24 in GAG/DNA (B) (P<0.05). Close homology between GAG/weight and GAG/DNA indicated that the results were not stemmed by either an increase or decrease in cell number.](image)

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Groups without IL-1β had significantly (P<0.001) higher GAG content than those with IL-1β. No significant differences were found among groups exposed to IL-1β or among groups without IL-1β exposure.

To test whether an increase or a decrease in cell numbers may be responsible for the differences in amount of measured GAG, total DNA in 3-D constructs was determined by diphenylamine assay. There was no statistically significant difference in DNA content among groups on any collection day (data not shown). Close similarities were noted between the results of GAG content, as measured by GAG/weight and GAG/DNA [Fig. 7(B)], suggesting that the differences between groups not exposed to IL-1β and groups exposed to IL-1β did not stem from either cell proliferation or cell death.

Because differences in GAG content among groups could be due to decreased production, increased degradation, or both, GAG concentrations in the liquid media were also examined by DMMB assay. No significant differences in GAG concentrations (µg/ml) in the liquid media were noted among groups, except on day 9 (P=0.038). GAG concentrations in the liquid media were significantly (P=0.015) lower in the IL-1β group than in control on day 9 (Fig. 8). To delineate if these findings were due to increased degradation or matrix turnover associated with GAG production, ratios of GAG concentrations in the liquid media to GAG in corresponding 3-D constructs were compared among groups. There were no significant differences in the ratios, GAG in the liquid medium:GAG in the 3-D, among groups (data not shown), indicating that GAG concentrations in the liquid media were reflective of matrix turnover of 3-D cultured chondrocytes. These results indicate that the addition of IL-1β inhibited GAG synthesis by chondrocytes in this system, and addition of either TIMP-1 or -2 did not effectively counteract this inhibition.

**HYDROXYPROLINE ASSAY**

The effects of IL-1β and TIMPs on collagen production were examined by HP assay. Both the groups contained measurable levels of HP in 3-D constructs at all sample times. HP content (HP/weight) was significantly (P<0.05)
Fig. 9. Mean (±S.E.M.) HP concentrations in 3-D constructs per dry weights. Concentrations of HP were measured on days 9, 15, and 24 of culture. HP contents (HP/weight) were significantly higher in the controls than in the T2 groups at each collection time (*P<0.05). In addition to the T2 group, IL-1β and IL/T2 had significantly lower HP contents than the control on day 24 (*P<0.05).

different among groups on each sample collection day. HP content in 3-D was significantly (P<0.05) higher in controls than the T2 group at each sample collection time. On day 24, in addition to the T2 group, groups IL-β and IL/T2 had significantly (P<0.05) lower HP content than controls (Fig. 9). Consistent with the results of collagen type II immunohistochemistry, HP content in the IL-1β group significantly (P<0.001) decreased between days 15 and 24. The results of HP assay in this study suggest that exogenous IL-1β and TIMP-2 had negative effects on collagen content.

Discussion

to date, four TIMPs, TIMP-1, -2, -3, and -4, have been identified in mammals. TIMPs are glycoproteins composed of two main domains: the N-terminal domain and the C-terminal domain, stabilized by six sulfide bonds. The inhibitory mechanism of TIMPs on MMPs is not fully understood. However, it has been reported that TIMPs inhibit MMP on a 1:1 basis by forming high-affinity complexes. Both domains, but mainly the N-terminal domain, are thought to contribute to the inhibitory activity against MMPs by forming a stable native molecule. Hughes et al. reported strong inhibition of MMP activity by both TIMP-1 and -2 in conditioned media of cultured porcine chondrocytes exposed to IL-1α. Concentrations of TIMP-1 (255 nM) and TIMP-2 (285 nM) in the present study were adapted from the study by Hughes et al., in which TIMPs used at these concentrations were reported to have strong inhibitory effects on MMPs. In the present study, neither TIMP-1 nor TIMP-2 appeared to prevent detrimental effects of IL-1β on canine chondrocytes cultured in 3-D agarose, despite evidence for decreases in MMPs in both the ECM and liquid media.

Although any in vitro model is inherently non-physiologic, the 3-D agarose culture system has been used for investigating characteristics of articular chondrocytes, as it permits maintenance of cell viability, differentiation, and appropriate ECM production. IL-1 has been used to create in vitro models of OA because of its known involvement in inciting and perpetuating this disorder. It is thought that IL-1, produced by polymorphonuclear leukocytes, synovial macrophages, synovial fibroblasts, or by chondrocytes themselves, stimulates the chondrocytes to synthesize proteases, such as MMPs and plasminogen activator, leading to cartilage degradation. Since it is likely that IL-1 initially exerts its effects on OA through access to the chondrocytes from the synovial fluid, it is added to the media of monolayer chondrocyte or explant cultures of articular cartilage to create an in vitro OA model. Cook et al. have described a 3-D culture system as an in vitro OA model that provides advantages for studying facets of OA. In this system, the 3-D constructs represent articular cartilage and the liquid media represent synovial fluid. Thus, at least a subset of cell, matrix, and synovial fluid changes that occur in OA can be investigated using this model. There was no direct evidence for penetration of exogenous TIMP-1 (28 kDa) and TIMP-2 (24 kDa) through agarose gel in the present study. However, the effects of recombinant proteins of similar molecular mass, including TGF-β (25 kDa), on chondrocytes in agarose have been reported. Although molecular mass is not the sole factor in determining penetration through agarose matrix, previous studies reported that TIMP-1 was secreted into culture media from chondrocytes cultured in agarose. The results of these studies suggest that TIMP can penetrate the agarose matrix. In addition, data from the present study indicate significant effects of TIMP-1 and -2 on agarose cultures, again suggesting that TIMP-1 and -2 can penetrate the agarose matrix and exert effects on the chondrocytes within the matrix. Therefore, a lack of blockage of detrimental effects of IL-1β by TIMPs, seen in the present study, cannot be attributed to the agarose matrix. Results of the present study indicate that the 3-D agarose culture system facilitates investigation of effects of a potential therapeutic intervention on chondrocytes in OA.

One can incriminate species differences with respect to the use of human recombinant TIMPs with canine chondrocytes as a reason for the fact that TIMPs did not prevent detrimental effects driven by IL-1β. However, it has been well documented that MMP and TIMP homologs in vertebrates are highly conserved. Amino acid sequence analyses of canine TIMP-1 and -2 show 79% homology with human TIMP-1 and 96% homologous with human TIMP-2, respectively. For MMPs, although the canine MMP-1 amino acid sequence is not present in the data bank (NCBI databases), those of canine MMP-3 (fragment) and MMP-13 are highly homologous with those of human MMP-3 (81%) and MMP-13 (98%), respectively. In addition, previouly reported data support cross-reactivity between human and canine MMPs. MMP-1, -3, and -13 have been localized in canine cartilage tissues by immunohistochemistry, using anti-human antibodies. Pelletier et al. reported imbalance between MMP and TIMP in joint tissues from a canine OA model in which MMP inhibition by TIMP was determined by an inhibitory assay between collagenase purified from human OA synovium and extracted canine TIMP. Based on these data and previous reports, a lack of significant effects of TIMP on chondrocytes exposed to IL-1β for some outcome measures in the present study cannot be completely attributed to species differences.

In the study reported here, cell viability and differentiation were maintained in the control group throughout the study period. The IL-1β groups showed less viable chondrocytes with lack of ECM formation more frequently than the
chondrocytes cultured without added IL-1β. Both histochemical and biochemical assessments revealed that GAG production was suppressed by the addition of IL-1β. This finding is consistent with a previous study that reported that IL-1β inhibited proteoglycan synthesis43. Addition of TIMP-1 or -2, at the single regimen evaluated, appeared to have minimal effects on histologic and histochemical characteristics of chondrocytes. Several functions of TIMPs other than inhibition of ECM degradation by MMPs have been reported, including inhibition of cell growth by preventing the degradation of growth factor-binding proteins by MMPs, blockage of cell cycle progression by upregulation of p27, and regulation of cell proliferation and apoptosis by binding to a putative cell-associated receptor44. No information, however, is present in the literature regarding effects of those TIMPs functions on chondrocytes, and their relevance with respect to the pathogenesis of OA is unclear. In the present study, TIMP-1 and -2 alone (without IL-1β) did not cause either an increase or a decrease in cell population, as determined by total DNA assay. It has been reported that the number of cells, as well as DNA content (3H-thymidine uptake), are largely unchanged in 3-D agarose culture over time26,45. It is possible that the slow cell proliferation rate in 3-D agarose concealed TIMP’s potential effects on cell proliferation. Cell viabilities in groups IL/T1 and IL/T2 were not inferior to those in the IL-1β group. However, 3-D constructs in these groups had less toluidine blue-stained matrix and lower GAG concentrations compared with those cultured without IL-1β. In the present study, we did not assess endogenous TIMP production and cannot describe the impact of exogenous TIMPs on endogenous TIMPs with clear evidence. However, largely unchanged results within each of non-IL-1β groups (control, TIMP-1, -2) and IL-1β groups (IL-1, IL/TIMP-1, IL/TIMP-2) imply that the impact of endogenous TIMPs on outcome measures used in this study is most likely negligible in the model used. Neither TIMP-1 nor TIMP-2 significantly alleviated the detrimental effects of IL-1β on chondrocytes or ECM in the present study.

In spite of the observation of morphologically less viable cells more frequently in the IL-1β group in this study, DNA assay did not show significant differences in DNA content among groups. This finding is consistent with the observation of monolayer-cultured chondrocytes in which IL-1β induces morphologic changes, including shrinking of the cells, whereas the IL-1β-induced morphologic changes are not associated with increased levels of apoptosis and cell death46. IL-1β is also known as an inhibitor of chondrocyte proliferation through activation of a nitric oxide-dependent prostaglandin E2 (PGE2) synthesis pathway47. However, the effect of IL-1β on cell proliferation in the present study could be insignificant because of the slow cell proliferation nature in 3-D agarose culture. Although it is possible that the percentage of cells that appeared less viable may not have been great enough to cause significant differences in total DNA content among groups, it is most likely that the cells that appeared less viable in the present study are not dead and the DNA is still measurable in those cells.

IL-1β caused significantly less GAG release to the liquid media when compared with controls on day 9 in this study, even in the presence of TIMP-1 or -2. This finding is contrary to a previous report in which addition of a high concentration of TIMP-1 to the culture media inhibited IL-1β-induced GAG release to the media of bovine cartilage explants22. In cartilage explants, IL-1β-induced GAG release was reported to be time-dependent48. Addition of IL-1β beginning on day 3 of 3-D culture may primarily be inhibitory for the formation of ECM rather than destructive to existing ECM. This may explain the lower release of GAG into the liquid media, concomitant with suppressed GAG production in 3-D constructs in the IL-1β-exposed groups. Data showing no significant differences in the ratio, liquid media GAG:corresponding 3-D GAG, substantiate this suggestion. Therefore, it is possible that the addition of TIMP-1 and -2 mildly inhibited IL-1β-induced matrix turnover suppression in the early period of the present study.

In contrast to the effects on proteoglycan synthesis and degradation, effects of IL-1β on collagen are reported to be less dramatic in vitro57. It has been reported that no collagen damage occurs in alginate-cultured chondrocytes after 2 days at 20 ng/ml IL-1β, and collagen release in bovine nasal cartilage explants occurs only after long time periods (>3 weeks) at relatively high concentrations of IL-1α (50 ng/ml)37,49. Our data regarding HP production and collagen type II immunoreactivity correspond to these findings37,49. In the present study, HP production and collagen type II immunoreactivity were minimally affected by the presence of IL-1β through day 15 of 3-D culture. However, both HP production and collagen type II immunoreactivity were decreased by IL-1β on day 24, and addition of TIMP-1 or -2 did not significantly alter the effects of IL-1β on collagen synthesis and degradation. Interestingly, addition of TIMP-2 alone (without IL-1β) resulted in significantly lower HP contents than controls at each sample collection time. A decrease in HP seen in the chondrocytes treated with TIMP-2 may be a result of as yet undefined functions of TIMP-2 that may lead to inhibition of collagen synthesis and/or enhancement of collagen degradation.

Maximal MMP synthesis by chondrocytes has occurred within a relatively short time period after the addition of IL-1β in previous studies9,37. Significantly higher MMP-3 concentrations and MMP-1 immunoreactivities in the IL-1β group in the early period of the present study agree with previous reports. Suppression of increased MMP levels by TIMP-1 and -2 in the present study also supports previously reported data regarding TIMP-mediated reduction in MMP activities5. However, the reduction in MMP levels seen in TIMP groups in this study did not translate into protection against the deleterious effects of IL-1β on chondrocytes and their ECM. IL-1β has been reported to induce chondrocytes to produce proteinases including MMPs and aggrecanases9. Although all MMPs present in cartilage (MMP-1, -2, -3, -7, -8, -9, -13, and MT1-MMP) are known to be capable of degrading the interglobular domain of the aggrecan core protein at the Asn341-Phe342, the role of MMPs in the catabolism of aggrecan is less clearly defined. Recently, the role of MMPs in aggrecan degradation has been shadowed by aggrecanases that cleave in the interglobular domain of aggrecan between G2 and G3 at four Glu1771-Ala1772, and Glu1871-Leu1872 (reviewed in references 52,53). A recent study by Malfait et al.54 demonstrated that aggrecan catabolism in OA cartilage was...
Inhibition of the detrimental effects of IL-1β in TNF-α models of arthritis. Based on these data and the present increase in inflammatory cytokines in a collagen-induced arthritis model, it is important to notice that proteoglycan degradation precedes collagen degradation15,37.

Interestingly, conflicting outcomes of adenovirus-based overexpression of TIMP-1 in two different murine arthritis models have been reported57,58. Schett et al.57 reported that clinical, radiographic, and histologic indicators of arthritis of tumor necrosis factor (TNF)α in transgenic mice were alleviated by adenoviral vector-based overexpression of TIMP-1, while serum levels of TNFα and IL-6 remained unchanged. However, Apparailly et al.58 reported that adenoviral vector-based overexpression of TIMP-1 was associated with deterioration in clinical, radiographic, and histologic measures of arthritis, concomitant with an increase in inflammatory cytokines in a collagen-induced model of arthritis. Based on these data and the present study, supraphysiologic levels of TIMP-1 may be beneficial in TNFα-driven arthritis, yet not in immune-mediated arthritis and this IL-1β-based OA model. Further elucidation of TIMP-mediated mechanisms that may lead to beneficial or unfavorable effects in various arthritides is necessary. In addition, it is apparent that the timing and amount of TIMP expression must be appropriately controlled in order to invoke beneficial effects in OA, while avoiding detrimental sequelae.

In conclusion, addition of TIMP-1 (255 nM) and TIMP-2 (285 nM) did not cause detrimental effects to chondrocytes in 3-D culture. Despite inhibition of MMP levels, TIMP-1 and -2 at the single concentration evaluated did not prevent histological and biochemical alterations in chondrocytes exposed to IL-1β. These data suggest that TIMP-1 and -2 may not be the main inhibitor to target for treatment of OA. Since most proteases including aggrecanases and reactive oxygen species produced by chondrocytes can be upregulated by elevated levels of IL-1 and TNFα, a lack of inhibition of the detrimental effects of IL-1β on chondrocytes in this study may be attributed to a lack of blockage of those catabolic factors by TIMPs. Regulation of the signals from these cytokines may have to be considered as a more colligative therapeutic modality for OA.

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