# TIMP-3 inhibits aggrecanase-mediated glycosaminoglycan release from cartilage explants stimulated by catabolic factors

Christi Gendron[a], Masahide Kashiwagi[a], Clare Hughes[b], Bruce Caterson[b], Hideaki Nagase[a,*]

[a] *Kennedy Institute of Rheumatology Division, Imperial College London, 1 Aspenlea Road, London W6 8LH, UK*
[b] *Connective Tissue Laboratories, Cardiff School of Biosciences, University of Cardiff, Cardiff CF10 3US, UK*

Received 22 August 2003; revised 15 October 2003; accepted 16 October 2003

First published online 12 November 2003

Edited by Veli-Pekka Lehto

**Abstract** **Aggrecanases are considered to play a key role in the destruction of articular cartilage during the progression of arthritis. Here we report that the N-terminal inhibitory domain of tissue inhibitor of metalloproteinases 3 (N-TIMP-3), but not TIMP-1 or TIMP-2, inhibits glycosaminoglycan release from bovine nasal and porcine articular cartilage explants stimulated with interleukin-1α or retinoic acid in a dose-dependent manner. This inhibition is due to the blocking of aggrecanase activity induced by the catabolic factors. Little apoptosis of primary porcine chondrocytes is observed at an effective concentration of N-TIMP-3. These results suggest that TIMP-3 may be a candidate agent for use against cartilage degradation.**


*Key words:* Interleukin-1; Retinoic acid; ADAMTS; Proteoglycan; Arthritis

## 1. Introduction

The aggregating proteoglycan, aggrecan, and collagen type II are the major components of the articular cartilage extracellular matrix. Diseases such as rheumatoid arthritis and osteoarthritis are associated with increased degradation of these macromolecular components [1,2]. This degradation is due to elevated proteolytic enzyme activities. Loss of aggrecan occurs initially, followed by mechanical failure of the tissue and collagen degradation [2]. Thus, aggrecan loss is considered to be a crucial early event in the progression of arthritic disease [3,4].

Two different groups of proteinases are thought to be involved in the degradation of aggrecan: matrix metalloproteinases (MMPs) and aggrecanases [3,4]. MMPs cleave the aggrecan core protein at the $Asn^{341}$–$Phe^{342}$ bond [5], whereas aggrecanases cleave the $Glu^{373}$–$Ala^{374}$ bond [6]. Aggrecanases are metalloproteinases that belong to the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family and four ADAMTSs (ADAMTS-1, -4, -5, and -9) have been shown to cleave aggrecan [7–10]. Both MMP- and aggrecanase-generated aggrecan fragments have been identified in cartilage explant cultures in vitro [11], as well as in human synovial fluid and cartilage tissues [12–15].

We have recently reported that tissue inhibitor of metalloproteinases 3 (TIMP-3) inhibits ADAMTS-4 and ADAMTS-5 with $K_i$ values in the subnanomolar range [16]. Hashimoto et al. [17] also reported similar results for ADAMTS-4 with TIMP-3. Therefore, we have investigated whether TIMP-3 can inhibit aggrecanases when added exogenously to cultured cartilage tissue explants. We chose two well-characterized model systems of aggrecan degradation for our study: bovine nasal and porcine articular cartilage explants stimulated with interleukin-1α (IL-1α) or retinoic acid [18,19]. This study demonstrates that N-terminal inhibitory domain of TIMP-3 (N-TIMP-3) inhibits aggrecan degradation in both cartilage explant systems, suggesting that TIMP-3 may potentially be used as a cartilage protective agent.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Materials were purchased from the following sources: Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin from Biowhittaker (Berkshire, UK); amphotericin B from Gibco (Paisley, UK); fetal calf serum from Labtech International (East Sussex, UK); chondroitinase ABC and keratanase from Seikagaku (Tokyo, Japan); anti-rabbit alkaline phosphatase (AP)-linked antibody, anti-mouse AP-linked antibody, and AP substrate (5-bromo-4-choloro-3-indolyl-1-phosphate and nitroblue tetrazolium) from Promega (Southampton, UK); dimethylmethylene blue (DMMB), shark chondroitin sulfate, retinoic acid, and type 1A bacterial collagenase from Sigma-Aldrich Company Ltd. (Dorset, UK); pronase E from BDH (Dorset, UK); propidium iodide/annexin V-fluorescein isothiocyanate conjugate (V-FITC) kit from Alexis Biochemicals (Nottingham, UK); and pre-stained Precision Protein Standards™ for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) from BioRad (Hemel Hempstead, UK). Recombinant N-TIMP-1 and N-TIMP-3 were expressed in *Escherichia coli* and folded from the inclusion bodies as described previously [16,20]. TIMP-2 was expressed in HEK 293/EBNA cells and purified from the conditioned media as described [21]. Monoclonal antibodies BC-3 (raised against the aggrecanase-generated aggrecan neoepitope ARGSV) and BC-14 (raised against the MMP-generated aggrecan neoepitope FFGVG) were generated and characterized as described previously [22]. Purified IL-1α was a gift from Professor J. Saklatvala (Imperial College London, UK). Bovine nasal septum and porcine metacarpophalangeal joints were supplied by Fresh Tissue (London, UK) and dissected on site within 24 h of slaughter.

### 2.2. Cartilage culture and inhibition studies

Porcine articular cartilage from the metacarpophalangeal joints of 3–9-month-old pigs was dissected into small shavings approximately

*Corresponding author. Fax: (44)-20-8356 0399.
*E-mail address:* h.nagase@imperial.ac.uk (H. Nagase).

*Abbreviations:* ADAMTS, a metalloproteinase with disintegrin and thrombospondin type-1 motifs; AP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; DMMB, dimethylmethylene blue; GAG, glycosaminoglycan; IL-1α, interleukin-1α; MMP, matrix metalloproteinase; N-TIMP, N-terminal inhibitory domain of tissue inhibitor of metalloproteinases; PBS, phosphate-buffered saline; SNP, sodium nitroprusside; TIMP, tissue inhibitor of metalloproteinases

0014-5793/03/$22.00 
doi:10.1016/S0014-5793(03)01295-X

3 mm long and 2–3 mm wide. Bovine nasal cartilage was dissected into small discs approximately 1 mm in diameter and 1–2 mm in height. After dissection, the cartilage was allowed to rest for 24 h at 37°C under 5% $CO_2$ in DMEM containing penicillin/streptomycin, amphotericin B, and 5% fetal calf serum. The medium was then refreshed and the cartilage was rested for a further 24–48 h. Each cartilage piece was placed in one well of a round bottom 96-well plate with 200 μl of serum-free DMEM with or without 10–100 ng/ml IL-1α or 1 μM retinoic acid and various concentrations of each TIMP. After 3 days, the conditioned media were harvested and stored at −20°C until use.

### 2.3. Primary chondrocyte isolation and culture

Porcine chondrocytes were isolated by digesting porcine articular cartilage with pronase E (1 mg/ml for 1 h at 37 °C) followed by bacterial collagenase (1 mg/ml for 5 h at 37°C). The cells were collected by passing the tissue through a cell strainer, washed twice with DMEM, and cultured in DMEM containing penicillin/streptomycin, amphotericin B, and 10% fetal calf serum. The next day, the cells were washed once with serum-free DMEM and then triplicate cultures were incubated in fresh media containing 0.5% fetal calf serum with or without each TIMP for 3 days. Chondrocyte viability and apoptosis were examined as described in Sections 2.6 and 2.7.

### 2.4. Analysis of glycosaminoglycan (GAG) release

GAG released into the conditioned medium was measured in duplicate using a modification of the DMMB assay as described by Farndale et al. [23]. Shark chondroitin sulfate (0–2.62 μg) was used as the standard. The percentage of total GAG released into the medium was calculated as follows: % of total GAG released = (total GAG in the medium)/(total GAG in the medium+total GAG remaining in the cartilage). Equivalent results were obtained irrespective of whether the data were analyzed as the percentage of total GAG released or the amount of GAG released into the conditioned medium. All data were analyzed by unpaired one-tail *t* tests with Welch's correction using the software package GraphPad Prism (San Diego, CA, USA).

### 2.5. Identification of aggrecanase- and MMP-generated aggrecan fragments by Western analysis

Aggrecan fragments released into the conditioned medium were deglycosylated using chondroitinase ABC and keratanase. The samples were then subjected to SDS–PAGE on 6% total acrylamide gels using the AMMEDIOL buffer system [24] and Western blotting analysis as described by Little et al. [19]. Pre-stained Precision Protein Standards™ from BioRad were used. The primary antibodies used to detect aggrecanase-generated and MMP-generated aggrecan fragments were BC-3 and BC-14, respectively [22]. Antigen–antibody complexes were detected by anti-mouse AP-linked donkey antibody and the AP substrate.

### 2.6. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Chondrocytes were seeded at $2.5\times10^4$ cells per well in a 96-well plate and treated as described in Section 2.3. Following the 3-day treatment, sterile-filtered MTT in phosphate-buffered saline (PBS) was added (0.5 mg/ml final concentration) and incubated for 4 h at 37°C. A final concentration of 5% SDS/5 mM HCl was then added to solubilize the formazan product and incubated overnight at 37°C in a shaking incubator. The absorbance was read at 590 nm.

### 2.7. Propidium iodide/annexin V staining of chondrocytes

Chondrocytes were seeded at $4\times10^5$ cells per well in a 48-well plate and treated as described in Section 2.3. Following the 3-day treatment, the cells were harvested from the plate by scraping. The cells were washed with PBS, spun, resuspended in 195 μl of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) and then reacted with 5 μl of annexin V-FITC for 10 min at room temperature. Next, the cells were washed with PBS, resuspended in binding buffer and reacted with propidium iodide at a final concentration of 2 μg/ml immediately before analysis using a Becton-Dickinson LSR flow cell cytometer.

## 3. Results

### 3.1. N-TIMP-3 inhibits IL-1α- and retinoic acid-stimulated aggrecan breakdown in cartilage explants

Bovine nasal cartilage explants were stimulated with IL-1α in the presence or absence of N-TIMP-1, TIMP-2, or N-TIMP-3 for 3 days. Explants treated with IL-1α alone showed an approximately five-fold increase in GAG release over controls (Fig. 1A). The IL-1α-stimulated release was significantly inhibited by the addition of N-TIMP-3 in a concentration-dependent manner. However, N-TIMP-1 and TIMP-2 were not effective even at 1 μM. Safranin O staining of the cartilage explants after treatment with IL-1α revealed

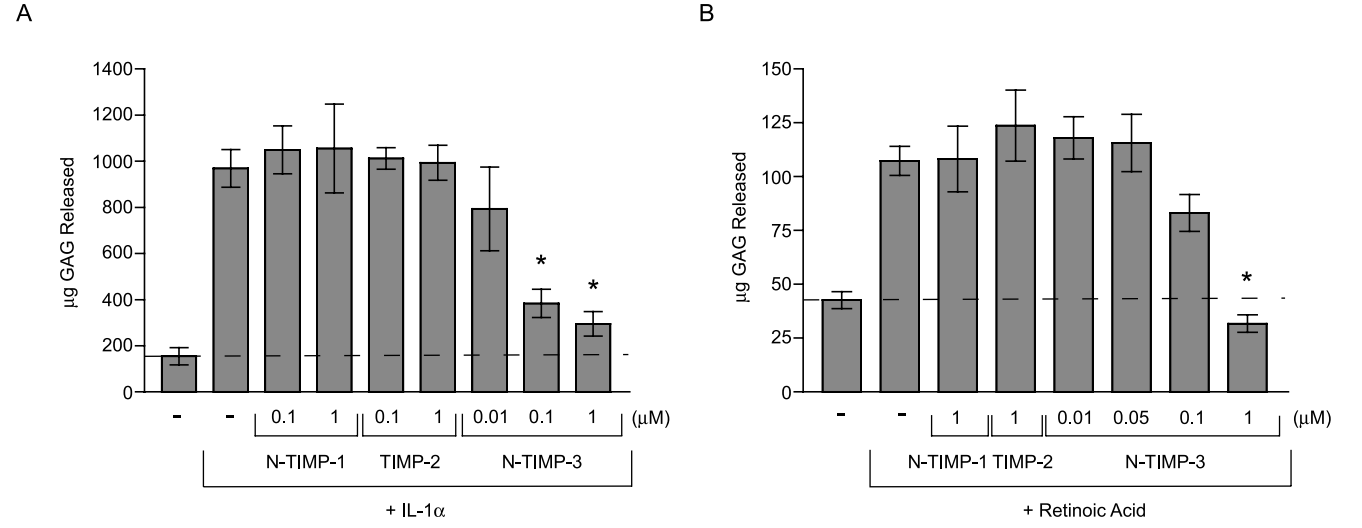


Fig. 1. N-TIMP-3 Inhibition of GAG release from cartilage stimulated with IL-1α or retinoic acid. A: Bovine nasal cartilage explants were treated with IL-1α (100 ng/ml) in the presence of TIMP for 3 days. The GAG released into the media was measured using the DMMB assay. The results are from three separate experiments ± S.E.M. ($n = 3$ for each experiment). B: Porcine articular cartilage explants were treated with 1 μM retinoic acid in the presence of TIMP for 3 days. The conditioned media were harvested and the released GAG was measured as above. The results are from three separate experiments ± S.E.M. ($n = 3$ for each experiment). *$P \leq 0.05$ as compared with the IL-1α or retinoic acid treatment without the inhibitor.

that the addition of N-TIMP-3 protected against the release of GAGs from the matrix (data not shown). Similar results were observed with IL-1α-stimulated porcine articular cartilage explants (data not shown).

The GAG release from porcine articular cartilage explants stimulated with retinoic acid was also inhibited by N-TIMP-3, but to a lesser extent compared with the IL-1α-stimulated cartilage (Fig. 1B). N-TIMP-1 and TIMP-2 did not inhibit retinoic acid-stimulated GAG release.

### 3.2. Aggrecanase activity is specifically inhibited by N-TIMP-3

Conditioned media from the experiments above were analyzed using monoclonal antibodies that recognize either the aggrecanase-generated aggrecan neoepitope ARGSV or the MMP-generated aggrecan neoepitope FFGVG. In concordance with the GAG release data, there was an increase in the amount of aggrecanase-generated aggrecan fragments released upon treatment with either stimulus (Fig. 2A,B). No MMP-generated fragments were detected (data not shown). The release of aggrecanase-generated fragments was partially inhibited by 0.05 μM N-TIMP-3 and completely blocked by 0.1 μM N-TIMP-3 in both IL-1α- and retinoic acid-stimulated cartilage (Fig. 2). N-TIMP-1 and TIMP-2 were not effective even at 1 μM.

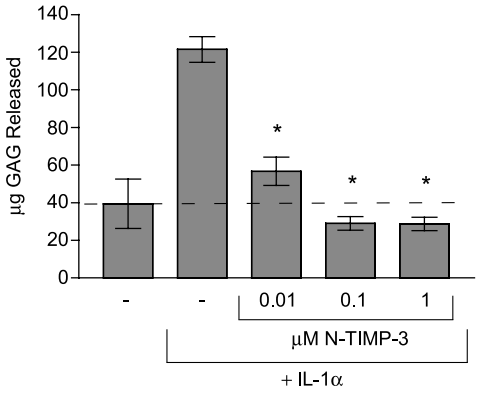


Fig. 3. Inhibition of GAG release by N-TIMP-3 after pre-treatment of cartilage with IL-1α. Porcine articular cartilage explants were treated with 10 ng/ml IL-1α for a period of 2 days. The conditioned media were then removed and replaced with fresh DMEM containing IL-1α plus various concentrations of N-TIMP-3 for further 3 days. The GAG released into the media was measured using the DMMB assay. The results are from a representative experiment ($n = 3$). *$P \leq 0.05$ as compared with the IL-1α treatment without the inhibitor.

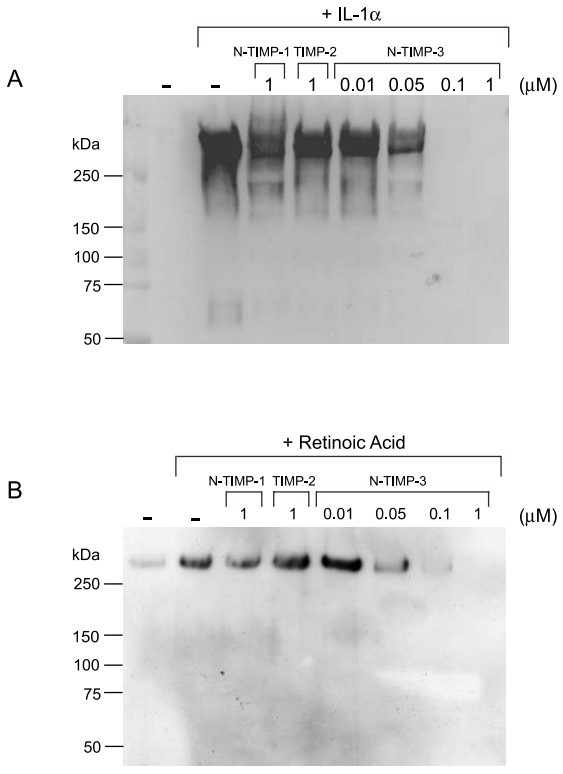


Fig. 2. Detection of aggrecanase-generated aggrecan fragments by the anti-ARGSV neoepitope antibody. A: Analysis of the conditioned media from bovine cartilage explants treated with IL-1α as in Fig. 1A. The samples, each containing 40 μg GAG, were deglycosylated and analyzed by Western blotting using an anti-ARGSV antibody as described in the Section 2.5. B: Analysis of the conditioned media from porcine articular cartilage explants treated with 1 μM retinoic acid as in Fig. 1B. The conditioned media, each containing 30 μg GAG, were deglycosylated and analyzed by Western blotting using an anti-ARGSV antibody as in A.

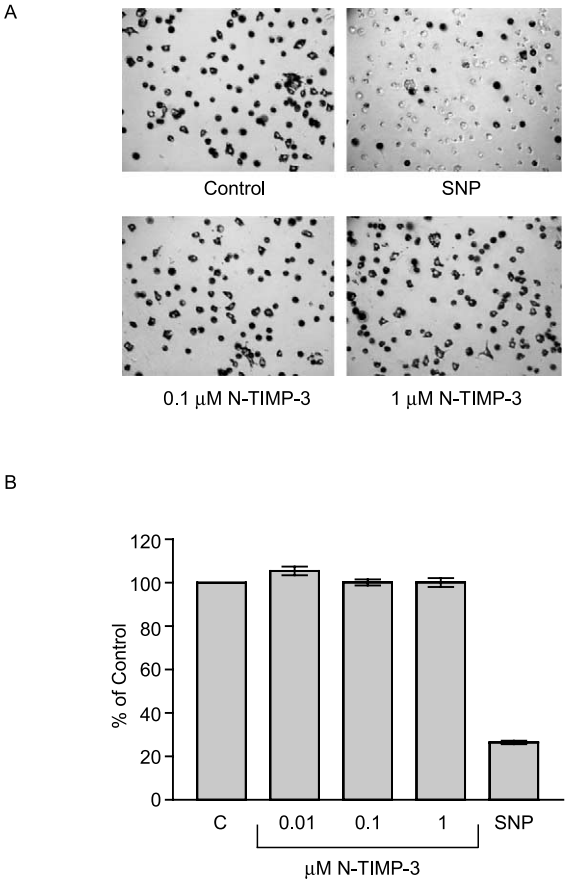


Fig. 4. Analysis of chondrocyte viability. Porcine articular chondrocytes were cultured in the presence of various concentrations of N-TIMP-3 for 3 days. The MTT assay was then carried out to test cell viability as described in Section 2.6. A: Cells following the incubation with MTT. B: The change in the absorbance of each treatment ($n = 3$).

### 3.3. Effect of N-TIMP-3 treatment before and after IL-1α stimulation of cartilage

To investigate the protective effect of TIMP-3, porcine cartilage explants were treated with 0.1 or 1 μM N-TIMP-3 for various periods of time prior to IL-1α stimulation. Each cartilage piece was then washed twice using serum-free DMEM and stimulated with IL-1α for 3 days in the absence of N-TIMP-3. The amount of GAG released into the conditioned media was subsequently measured. When 0.1 μM N-TIMP-3 was added, about 50% inhibition was observed after a 30-min pre-incubation and 72% after a 24-h pre-incubation. In the case of 1 μM N-TIMP-3, a 30-min pre-incubation was sufficient to give $>70\%$ inhibition, and complete inhibition was achieved with an 8-h pre-incubation. This indicates that the cartilage adsorbs N-TIMP-3 reasonably rapidly, and the continuous presence of N-TIMP-3 in the medium during the 3-day IL-1α stimulation is not necessary.

To further test the effectiveness of TIMP-3 protection against cartilage degradation, porcine cartilage explants were first treated with 10 ng/ml IL-1α for 2 days and then 0.01, 0.1 or 1 μM N-TIMP-3 with IL-1α for 3 days. Each N-TIMP-3 concentration was able to significantly inhibit further GAG release (Fig. 3). In these cases, N-TIMP-3 was slightly more effective as compared to the earlier experiments where the inhibitor and IL-1α were added together from the beginning.

### 3.4. Effect of N-TIMP-3 on chondrocyte apoptosis

TIMP-3 causes apoptosis in a variety of cell types including rat vascular smooth muscle, HeLa, colon carcinoma, and melanoma cells [25–27]. We therefore examined whether the inhibition of GAG release from stimulated cartilage explants by N-TIMP-3 was due to chondrocyte apoptosis. For this purpose, primary porcine chondrocytes were incubated with various concentrations of N-TIMP-3 for a period of 3 days. The cells were then assessed for cell viability using MTT (Fig. 4) or for apoptosis using propidium iodide/annexin V-FITC staining (Fig. 5). As a positive control for cell death, chondrocytes were treated with sodium nitroprusside (SNP). The MTT assay indicated that overall cell viability did not change in the presence of N-TIMP-3 at concentrations up to 1 μM. Propidium iodide/annexin V-FITC staining showed that there was no significant increase in chondrocyte apoptosis up to 1 μM N-TIMP-3 (Fig. 5). Other cell death assays measuring caspase activation and TUNEL staining confirmed these results (data not shown).

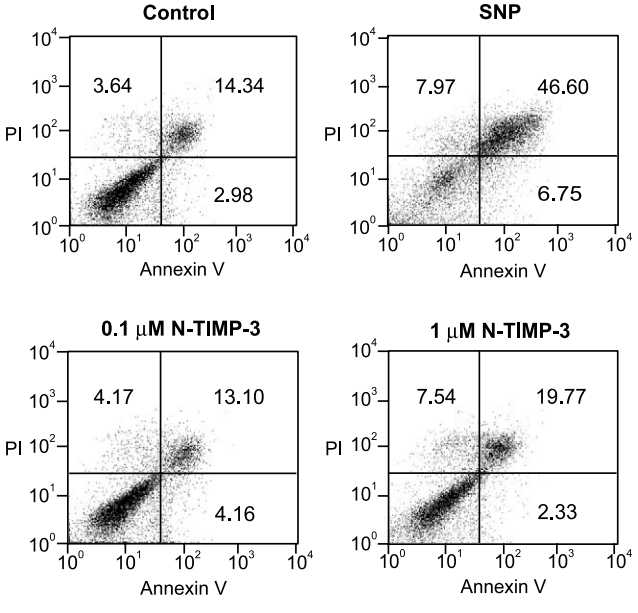


Fig. 5. Analysis of chondrocyte apoptosis. Porcine articular chondrocytes were incubated with various concentrations of N-TIMP-3 for 3 days. The cells were harvested, stained with propidium iodide and annexin V-FITC, and analyzed using flow cytometry. The number in each quadrant indicates the percentage of cells in that area ($n=3$). The lower right and upper right quadrants indicate the percentage of chondrocytes in early and late apoptosis, respectively.

## 4. Discussion

Previous studies have shown that TIMP-3 is a potent inhibitor of ADAMTS-4 and ADAMTS-5 with an apparent $K_i$ of 3.3 and 0.66 nM, respectively [16,17]. In this report, we have provided evidence that TIMP-3 effectively prevented the breakdown of aggrecan in bovine nasal and porcine articular cartilage explants stimulated by IL-1α or retinoic acid. The use of neoepitope antibodies to identify proteolytic fragments of the aggrecan core protein indicated that aggrecanase-mediated degradation of aggrecan was inhibited by N-TIMP-3.

N-TIMP-1 and TIMP-2 inhibit MMPs with low nanomolar $K_i$ values, but their inhibitory activities for aggrecanases are weak. The $IC_{50}$ value of TIMP-1 for aggrecanase activity from bovine cartilage was reported to be 210 nM [18] and those of TIMP-1 and TIMP-2 for ADAMTS-4 are 350 and 420 nM, respectively [17]. Bonassar et al. [28] reported a partial inhibition of GAG loss from IL-1β-treated bovine cartilage by 4 μM TIMP-1. These authors also estimated that approximately 10% or less TIMP-1 (~400 nM) is incorporated into the tissue based on their measurements of partitioning TIMP into adult cartilage [28]. This concentration is similar to the $IC_{50}$ values reported for TIMP-1 inhibition of aggrecanase activity. It is not clear what proportions of N-TIMP-1 and TIMP-2 in the medium penetrate the cartilage, but their concentrations would be sufficient to inhibit most MMPs given their low to subnanomolar inhibition constants [29]. Lack of inhibition of the stimulated GAG release by TIMP-1 and TIMP-2 therefore suggests that MMPs are not involved in aggrecan degradation during the culture period we examined. These observations agree well with those reported by Little et al. [11] indicating that MMPs do not participate in aggrecan degradation until after 3 weeks of culture.

Currently four ADAMTSs (ADAMTS-1, -4, -5, and -9) have been shown to digest aggrecan core protein at the $Glu^{373}$–$Ala^{374}$ bond, a typical aggrecanase site [7–10]. While it is yet to be investigated which of the ADAMTSs are the major enzymes involved in aggrecan degradation in these two models of cartilage catabolism, our TIMP-3 inhibition studies suggest that there are at least two pathways for GAG release: one that is aggrecanase dependent and the other that is aggrecanase independent. Western blotting with the anti-ARGSV antibody indicated a clear dose-dependent inhibition of aggrecanase activity by TIMP-3 in both IL-1α- and retinoic acid-stimulated cartilage, but they did not exactly match TIMP-3 inhibition of GAG release (see Figs. 1 and 2). This difference was more prominent in cartilage stimulated with retinoic acid than with IL-1α-treated cartilage. For example, 0.05 μM of TIMP-3 exhibited little inhibition of GAG release

from retinoic acid-stimulated cartilage, but it effectively inhibited aggrecanase activity. A possible mechanism of aggrecanase-independent GAG release may be due to the action of hyaluronidases as proposed by Sztrolovics et al. [30,31].

Pre-treatment of cartilage with 1 μM TIMP-3 for a minimum of 30 min followed by removal of the inhibitor before IL-1α-stimulation is sufficient to significantly inhibit aggrecan catabolism. This is most likely due to TIMP-3 binding to the cartilage matrix. Factors that may influence the ability of N-TIMP-3 to enter the cartilage matrix include its basic p*I* of 9.27 and its affinity for polyanionic components of the extracellular matrix [32]. Even more effective inhibition of GAG release was observed by adding N-TIMP-3 to the cartilage after 2 days of stimulation with IL-1α. The reason for this observation is not clear, but it may be that N-TIMP-3 can penetrate the tissue more readily when the cartilage matrix is partially degraded and therefore block aggrecanase activity more effectively.

A number of studies have indicated that TIMP-3 causes apoptosis of several cell types [26,33,34], while TIMP-1 and TIMP-2 have anti-apoptotic activity [35–37]. N-TIMP-3-induced apoptosis correlates with the stabilization of TNFα, Fas, and TNFα-related apoptosis inducing ligand receptors on the cell surface [26,33,34]. It is also dependent on Fas-associated death domain [38]. In our study on isolated primary chondrocytes, no significant apoptosis was observed even at 1 μM N-TIMP-3. Therefore, inhibition of aggrecan breakdown by N-TIMP-3 was due solely to the inhibition of metalloproteinases.

Various anti-rheumatic compounds may enhance the production of TIMP-3 as a mechanism of disease relief. For example, calcium pentosan polysulfate has been shown to relieve the symptoms of osteoarthritis [39] and Takizawa et al. have demonstrated that the addition of calcium pentosan polysulfate to human rheumatoid synovial fibroblasts increased TIMP-3 protein levels without an increase in mRNA levels [40]. Furthermore, the addition of calcium pentosan polysulfate was shown to inhibit aggrecan catabolism in bovine articular cartilage explants [41]. Heparinoid, a component of the anti-rheumatic drug Mobilat, was also shown to increase the amount of TIMP-3 protein in the presence of IL-1α via a mechanism that was independent of mRNA levels [42]. Other work has demonstrated that the adenoviral expression of TIMP-3 in rheumatoid synovial fibroblasts significantly inhibits the invasion of the pannus into the cartilage matrix [43]. These studies support the notion that TIMP-3 plays a pivotal role in ameliorating arthritis. The data reported herein provides direct evidence that TIMP-3 protects the articular joint against cartilage degradation. Therefore, TIMP-3 can be considered as a potential chondroprotectant in cartilage degenerative diseases such as osteoarthritis.

*Acknowledgements:* This work was supported by the Wellcome Trust Grant 057508 and NIH Grant AR40994 (to H.N.). C.G. is the recipient of a Wellcome Trust Prize Studentship. C.H. is an ARC Fellow.

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