Osteoarthritis and Cartilage

International Cartilage Repair Society

ICRS

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Intra-articular injection of tumor necrosis factor-α in the rat: an acute and reversible *in vivo* model of cartilage proteoglycan degradation

A. M. Malfait*, M. Tortorella, J. Thompson, R. Hills[a], D. M. Meyer, B. D. Jaffee[b], K. Chinn[c], N. Ghoreishi-Haack, S. Markosyan[d] and E. C. Arner
Pfizer Global Research and Development, St Louis Laboratories, 700 Chesterfield Parkway, St Louis, MO 63017, United States

Summary

Objective: To develop an *in vivo* model for rapid assessment of cartilage aggrecan degradation and its pharmacological modulation.

Design: Tumor necrosis factor-α (TNFα) was injected intra-articularly (IA) in rat knees and aggrecan degradation was monitored at various times following challenge. Articular cartilage was assessed for aggrecan content by Safranin O staining and by immunohistochemistry for the NITEGE epitope. Synovial fluids (SFs) were analyzed for sulfated glycosaminoglycans (GAGs) using the dimethylmethylene blue dye assay and for aggrecan fragments generated by specific cleavage at aggrecanase-sensitive sites by Western blot analysis with neoepitope antibodies. Indomethacin, dexamethasone, and an aggrecanase inhibitor were evaluated for their ability to modulate TNFα-induced proteoglycan degradation *in vivo*.

Results: (1) IA injection of TNFα in the knee joint of rats resulted in transient aggrecan degradation and release of aggrecanase-generated aggrecan fragments from the articular cartilage into the SF; (2) a correlation was observed between histologically assessed depletion of aggrecan from the articular cartilage and the appearance of specific neoepitopes in the SF; (3) aggrecan degradation was inhibited by an aggrecanase inhibitor as well as by dexamethasone, but not by the non-steroidal anti-inflammatory drug (NSAID), indomethacin.

Conclusion: TNFα injection in the knee joints of rats results in rapid transient cartilage proteoglycan degradation, mediated by cleavage at the aggrecanase sites. Biomarker read-out of specific neoepitopes in the SF enables the use of this mechanism-based model for rapid evaluation of aggrecanase-mediated aggrecan degradation *in vivo*.
© 2008 Osteoarthritis Research Society International. Published by Elsevier Ltd. All rights reserved.

Key words: Aggrecanase, ADAMTS, Aggrecan, Cartilage, Animal model, Neoepitope.

Introduction

Osteoarthritis (OA) is characterized by the loss of the aggregating proteoglycan, aggrecan, from articular cartilage. In healthy cartilage, aggrecan, with its negative charge and high water-binding capacity, provides the tissue with its properties of compressibility and resilience. In human OA, as in most animal models that resemble human disease, aggrecan loss from the cartilage matrix is an early feature of pathology[1,2]. Aggrecan depletion compromises cartilage function and facilitates the progressive loss of collagen, fibrillation, and ultimate destruction of the cartilage[3].

Loss of aggrecan is the result of enzymatic degradation of the aggrecan core protein by aggrecanases, a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-4[4] and ADAMTS-5[5−9] (reviewed in Ref. 10). It was recently shown that ADAMTS-5 knock-out (KO) and ADAMTS-4/-5 double KO, but not ADAMTS-4-KO mice are resistant to surgically induced OA, suggesting that ADAMTS-5 is the major aggrecanase in murine arthritis[11−14]. Interestingly, ADAMTS-5-deficient mice are protected not only against cartilage erosion, but against the entire subsequent OA pathology, including osteophyte formation and subchondral bone sclerosis[15]. It is still being debated which aggrecanases are the key players in human disease, but siRNA studies suggest a contribution of both ADAMTS-4 and ADAMTS-5 in human cartilage explants[16]. A critical role of the aggrecanases in the osteoarthritic process is evident and thus inhibitors of these proteases have potential for treatment of OA.

Numerous animal models that mimic aspects of the human disease have been developed. Spontaneous arthritis in aging animals, or surgically, mechanically, and chemically induced models, as well as genetically modified mice have been described (reviewed in Refs. 17,18). Appropriate *in vivo* models are essential to gain insight concerning mediators and pathways involved in joint destruction, and to evaluate pharmacological modulation of these pathways. Since OA is a chronic progressive disease, a relevant animal model should demonstrate relatively slow progression,

[a]Current address: Johnson and Johnson, Exton, PA 19341, United States.

[b]Current address: Novartis Institute for Biomedical Research, Inc., Cambridge, MA 02139, United States.

[c]Current address: Cellular Bioengineering, Inc., Honolulu, HI 96826, United States.

[d]Current address: Abbott Laboratories, Libertyville, IL 60048, United States.

*Address correspondence and reprint requests to: A. M. Malfait, Pfizer Global Research and Development, St Louis Laboratories, 700 Chesterfield Parkway West, St Louis, MO 63017, United States. Tel: 1-636-247-7859; E-mail: anne-marie.malfait@pfizer.com

particularly for evaluating disease modifying OA drugs (DMOADs). However, the long time-course of disease development in these models, along with time-intensive histological endpoints, precludes evaluating large numbers of potential therapeutics for efficacy. Thus, a need exists for animal models of acute cartilage degradation that will permit assessment of novel inhibitors for mechanism-related efficacy and potency *in vivo*, prior to evaluation in a more relevant, but slow-developing chronic OA model. Ideally, such a model should allow for a cartilage biomarker read-out, in order to avoid dependence on labor- and time-intensive histology as a primary outcome measure.

Here, we demonstrate that (1) intra-articular (IA) injection of tumor necrosis factor-α (TNFα) in rat knee joint results in transient aggrecan degradation and release of aggrecanase-generated aggrecan fragments from the articular cartilage into the synovial fluid (SF); (2) there is a correlation between histologically assessed depletion of proteoglycans from cartilage and the appearance of specific neoepitopes in the SF that allows for a fast biomarker read-out; (3) aggrecan degradation in this model is inhibited by an aggrecanase inhibitor, as well as by dexamethasone, but not the NSAID, indomethacin, which is ineffective as a cartilage protectant in man. Collectively, these data suggest that IA TNFα injection in rat knees provides a mechanism-based animal model of cartilage degradation that is suitable for rapid assessment of aggrecan cleavage and putative chondroprotective agents *in vivo*.

Materials and methods

MATERIALS

Recombinant human (rh) TNFα was used, because there is a high degree of homology between rat and human TNFα, and rat cells respond to rhTNFα. rhTNFα containing an N-terminal his-tag was expressed and purified at Pfizer. The construct has a pI of 7.4, and great care was taken to formulate the purified cytokine in PBS at pH8.5, in order to avoid aggregation and inactivation during storage and injection. Biological activity of TNFα was confirmed by testing its ability to induce aggrecan release from bovine nasal cartilage explants, as described for interleukin-1 (IL-1)[19] (not shown). Endotoxin levels were determined by a turbidimetric method, and were below detectable levels of 0.016 EU. The monoclonal neoepitope antibody (Ab), BC-3, that recognizes the new N-terminus, [374]ARG, on aggrecan fragments produced by cleavage at the Glu^{373}−Ala^{374} bond was licensed from Dr. Caterson (Cardiff, Wales)[20]. Although BC-3 was raised to the human sequence (ARGS), the Ab recognizes the rat sequence (ARGN). The polyclonal neoepitope Abs that recognize the N-terminus [1565]AGEG on aggrecan fragments generated by cleavage at the Glu^{1564}−Ala^{1565} bond and the C-terminus $SELE^{1293}$ on fragments generated by cleavage at Glu^{1274}−Gly^{1275} were developed, as described in Ref. 21. The polyclonal Ab against $NITEGE^{373}$, the C-terminus generated when aggrecanase cleaves aggrecan at the Glu^{373}−Ala^{374} bond, and the neoepitope Ab, AF-28, that recognizes the new N-terminus FFGV on fragments produced by cleavage at the Asn^{341}−Phe^{342} bond[22], were a gift from Dr. Amanda Fosang (University of Melbourne, Australia). Chondroitinase ABC lyase (*Proteus vulgaris*), keratanase (*Pseudomonas* sp.) and keratanase II (*Bacillus* sp.) were from Seikugoku (Kogyo, Japan). Hyaluronate lyase from Streptomyces hyalurolyticus, papain, indomethacin, and (soluble) dexamethasone were from Sigma, St. Louis, MO. A selective aggrecanase inhibitor, reported by researchers at DuPont Pharmaceuticals, "compound 11", a (2R)-N⁴-hydroxy-2-(3-hydroxy-benzyl)-N¹-[(1S,2R)-2-hydroxy-2,3-dihydro-1H-inden-1-yl]butanediamide derivative[23], was synthesized at Pfizer. We assessed compound 11 in ADAMTS-4/-5 and matrix metalloproteinase (MMP) enzymatic assays[9] and determined that it inhibited ADAMTS-4 and ADAMTS-5 with a K_i of 22.5 and 36.2 nM, respectively, while sparing MMP-1, MMP-2, MMP-3, MMP-13, and MMP-14 ($K_i > 10,000$, 6170, 6120, 5950, and $>10,000$, respectively). This compound blocked IL-1-induced aggrecanolysis in bovine nasal cartilage[19] with an IC_{50} of 370 nM and an IC_{90} of 4 μM.

IN VIVO PROTOCOLS

Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN), weighing 300 g, received an IA injection containing TNFα in a volume of 20 μl saline/0.1% bovine serum albumin (BSA). Rats injected with 20 μl saline/0.1% BSA alone served as vehicle controls. Pilot studies using Indian ink asserted that a 20-μl instillate was confined to the synovial space, and no leakage from the joint occurred (not shown). In addition, pilot dose−response studies were performed, where rats were injected IA with 0.01−20 μg TNFα and euthanized 16 h later for measurement of glycosaminoglycan (GAG) release into the SF. TNFα doses between 0.1 and 20 μg resulted in SF GAG levels greater than in vehicle control joints, with maximal release achieved with 5 μg TNFα and a plateau reached at higher doses. Based on this, 10 μg TNFα were used for all studies described herein. For time-course studies, $n = 6$−8 animals/group were euthanized at various times following challenge with 10 μg TNFα or vehicle. At necropsy, knee joints were lavaged with 100 μl saline using an insulin syringe. Lavage fluids were spun and frozen at −20°C. Knee joints were trimmed of muscle and connective tissue and collected in 10% neutral buffered formalin for histology.

INHIBITOR STUDIES

Drugs or vehicles were administered immediately prior to IA TNFα injection. Indomethacin and dexamethasone were administered p.o. in saline/0.1% Tween 80. For intravenous (IV) studies, soluble dexamethasone was administered in saline. The aggrecanase inhibitor was administered in an IV infusion, in a vehicle consisting of 30% PEG400, 65% of 5% D-mannitol, and 5% 0.1 N NaOH.

JOINT HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Following 6 days in 5% formic acid decalcifier, joints were cut into halves in the frontal plane and paraffin-embedded. Three sections were cut from each knee at 200 μm-steps and stained with Safranin O for evaluation of proteoglycan content. A score was assigned based on intensity of Safranin O staining by an observer blinded to the treatments received. Scores were 2, 1.5, 1, 0.5, or 0 for staining intensities ranging from full staining intensity (2) to total loss of staining (0). The medial and lateral femoral condyles and tibial plateaus were each scored separately. Total joint score was the sum of these four scores and thus the maximal overall joint score was 8. Data were analyzed using Mann−Whitney *U* test for unpaired nonparametric data.

Slides for immunohistochemistry were deparaffinized, rehydrated through graded ethanol, pretreated with chondroitinase ABC (0.1 U/ml), washed in TBS−Tween, and unspecific antibody binding blocked using Protein Block. Slides were incubated with primary antibody (NITEGE) at a 1/250 dilution. Antibody binding was detected using the Envision Plus Alkaline Phosphatase system (DAKO), using BCIP/NBT (Roche) as the substrate, in the presence of levamisole to block endogenous alkaline phosphatase. Negative controls contained no primary antibody but rabbit serum at an equivalent protein concentration.

GAG ASSAY

SF lavage samples were treated with hyaluronidase, 50 U/ml, at room temperature until the sample was liquefied. Samples were centrifuged, supernatants collected and an equal volume of 500 μg/ml papain was added (final concentration 250 μg/ml). Samples were incubated for 2 h at 65°C, cooled to room temperature and spun[24]. GAG levels in SF were assessed with 1,9-dimethylmethylene blue (DMMB), using shark chondroitin sulfate as a standard[25]. Results are reported as μg GAG per ml lavage fluid.

NEOEPITOPE WESTERN BLOT ANALYSIS

For analysis of aggrecan fragments generated by specific cleavage at the aggrecanase cleavage sites, Glu^{373}−Ala^{374}, Glu^{1274}−Gly^{1275}, and Glu^{1564}−Ala^{1565}, aggrecan metabolites in lavage fluids were enzymatically deglycosylated with chondroitinase ABC (0.1 U/10 μg GAG) plus keratanase (0.1 U/10 μg GAG)/keratanase II (0.002 U/10 μg GAG) for 4 h at 37°C in buffer containing 50 mM sodium acetate, 0.1 M Tris/HCl, pH 6.5 (equal volumes of lavage fluid and buffer with deglycosylating enzymes). After digestion, samples were concentrated using a MicroCon concentrator (Millipore, Bedford, MA) to a final volume of 10 μl, loaded on five-well 4−12% gradient gels, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, transferred overnight to nitrocellulose and immunolocated with 1:1000 dilution of the specified antibody, as described in Ref. 9. Subsequently, membranes were incubated with goat anti-rabbit IgG alkaline phosphatase conjugate and aggrecan catabolites visualized by incubation with the appropriate substrate (Promega Western blot alkaline phosphatase system). Reactive aggrecan fragments were quantified by scanning densitometry.
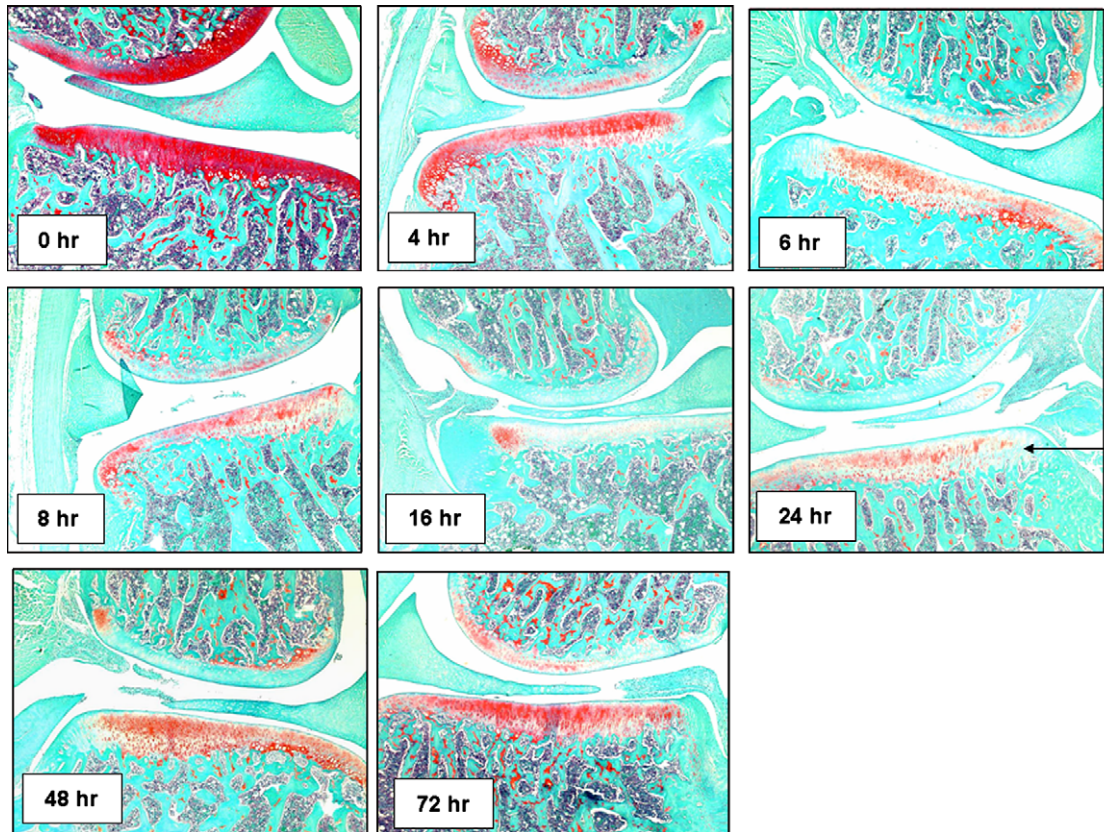
Fig. 1. Time-course of cartilage aggrecan depletion. Rats were injected IA with 10 μg TNFα. At various times following challenge, groups of rats were euthanized at the specified time-points and the knee joints were collected for Safranin O staining. "Time 0" represents uninjected controls. Maximal depletion of aggrecan occurred 16 h after challenge, followed by partial replacement of proteoglycans, first in the tibial plateau (arrow, 24 h) and later in the femoral condyles. Each rat knee shown is representative for a group of $n = 6$.

Results

TNFα TRIGGERS RAPID AGGRECAN DEPLETION OF CARTILAGE

Joints were taken for histology at various times following IA TNFα and stained with Safranin O (Fig. 1). Compared with staining of normal knee joints (time 0), a decrease in Safranin O staining was observed as early as 4 h following TNFα administration. Loss of staining continued progressively over time, first in the femoral condyles, and then in the tibial plateaus, and was maximal by 16 h after TNFα. At this time, loss of Safranin O staining in articular cartilage was nearly complete, while fast green stain revealed an intact collagen network (no fibrillation at the cartilage surface was observed). By 24 h following challenge, Safranin O staining began to show gradual recovery of proteoglycans, first in the tibial plateau, and later in the femoral condyles. Staining continued to increase through 72 h. Figure 2 compares a TNFα-injected knee at the height of aggrecan depletion, 16 h after TNFα injection, with an uninjected knee. Significant proteoglycan depletion from the cartilage of TNFα-injected knees, as monitored by Safranin O staining [Fig. 2(A)], was accompanied by generation of the NI-TEGE[373] neoepitope [Fig. 2(B)], indicating aggrecanase-mediated cleavage at the Glu[373]/Ala[374] bond within the IGD of aggrecan.

In order to enable statistical analysis, an additional study was performed where joints from rats euthanized 6 h following IA TNFα or saline were stained with Safranin O and proteoglycan loss assessed by a blinded observer. Table I shows that TNFα provoked a significant loss of proteoglycans from the knee cartilage ($P < 0.05$ for total score). The aggrecan loss was greatest in the femoral condyles ($P = 0.007$ for the lateral and $P = 0.019$ for the medial condyle).

TIME-COURSE OF APPEARANCE OF AGGRECAN FRAGMENTS IN SF

To gain a better understanding of how the kinetics of TNFα-induced aggrecan depletion correspond to the kinetics of aggrecan fragment release from cartilage, groups of animals were euthanized at various times following challenge and synovial lavage fluids were analyzed for GAG content or were pooled and subjected to Western blot analysis for the ARG neoepitope on aggrecan fragments generated by cleavage at Glu[373]/Ala[374]. IA TNFα resulted in a time-dependent release of GAG into the SF [Fig. 3(A)]. Levels were increased compared to vehicle-injected controls by 6 h following challenge and reached maximal levels very rapidly, between 8 and 16 h. By 24−48 h, levels fell dramatically, returning to control levels. Analysis of these same samples by
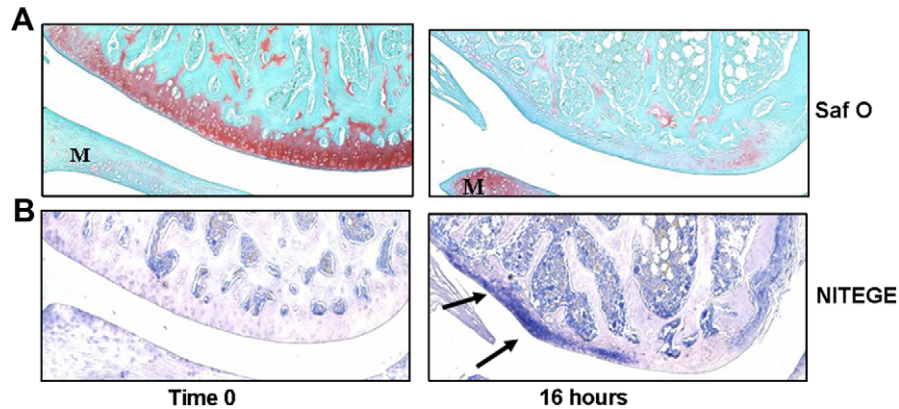
Fig. 2. Assessment of aggrecanase-generated NITEGE neoepitope. Rats were injected IA with 10 μg TNFα, and 16 h later, knee joints were collected for histology compared with time 0 (uninjected controls). (A) Safranin O staining shows proteoglycan depletion in the femoral condyle; (B) immunohistochemistry reveals the presence of the NITEGE neoepitope in the depleted areas (blue signal, arrows). M = meniscus.

Western blot detected the presence of a 250-kDa ARGN-containing fragment in response to TNFα. Quantitation by scanning densitometry showed that the ARGN epitope in SF increased with time between 6 and 16 h following challenge [Fig. 3(B)]. After 24 h, immunoreactive fragments were no longer detectable. No ARG-reactive fragments were detected in the fluids from vehicle controls at any of the time-points. These data demonstrate a correlation of GAG release and generation of the ARGN neoepitope over time, and provide further evidence that the model is aggrecanase-dependent.

EFFECT OF ANTI-INFLAMMATORY DRUGS ON AGGRECAN DEGRADATION

Rats were dosed orally with indomethacin (10 mg/kg) or dexamethasone (1 mg/kg), or with vehicle as a control, immediately prior to IA TNFα, and euthanized 16 h later. Animals injected IA with saline served as negative controls. Indomethacin did not significantly inhibit GAG release into the SF in response to TNFα, whereas dexamethasone caused essentially complete inhibition (Fig. 4).

To examine the dose−response for inhibition, rats were injected IA with TNFα or vehicle. Immediately following challenge, animals were treated by continuous IV infusion for 16 h with various concentrations of dexamethasone or with vehicle control. After 16 h, rats were euthanized and

joints lavaged. A dose-related inhibition of GAG release into the SF was observed [Fig. 5(A)]. Maximal inhibition (94%) was obtained with 0.1 mg/ml and the ED_{50} for inhibition of TNFα-induced GAG release was estimated from the dose−response curve to be 0.006 mg/ml. Dexamethasone also caused a dose-dependent inhibition of ARG neoepitope generation in the SF [Fig. 5(B)]. The ED_{50} for inhibition of ARG-generation was between 0.001 and 0.01 mg/ml dexamethasone, similar to that for inhibition of GAG release. In order to evaluate the correlation between inhibition of GAG release and inhibition of ARG neoepitope generation, Western blots from several separate studies were quantified by scanning densitometry. Percent inhibition of GAG release was plotted vs percent inhibition of ARG-generation [Fig. 5(C)], revealing a strong positive correlation between these two parameters.

ASSESSMENT OF OTHER AGGRECAN NEOEPITOPES IN SFS

In addition to cleavage within the interglobular domain at the $Glu^{373}−Ala^{374}$ bond, aggrecanases cleave the aggrecan core protein at four sites located in the chondroitin sulfate-rich region, between G2 and G3, at Glu^{1293}/Gly^{1294}, Glu^{1478}/Gly^{1479}, Glu^{1584}/Ala^{1585}, and Glu^{1683}/Leu^{1684}. Because these have been shown to be preferred cleavage sites in bovine and human aggrecan[21,26], it can be expected that these epitopes will appear in SF before ARG-

Table I

Proteoglycan staining in rat knees, 6 h following IA administration of 10 μg TNFα. A score was assigned based on intensity of Safranin O staining by an observer blinded to the treatments received. Scores were 2, 1.5, 1, 0.5, or 0 for staining intensities ranging from full Safranin O staining intensity (2) to total loss of staining (0). The medial and lateral femoral condyles and tibial plateaus were each scored separately. The total joint score was the sum of these 4 scores and thus the maximal overall joint score was 8. Data were analyzed using Mann−Whitney U test for unpaired nonparametric data. Values shown are the median, with the range shown in parentheses. P-values were calculated using a two-sided Mann−Whitney U test

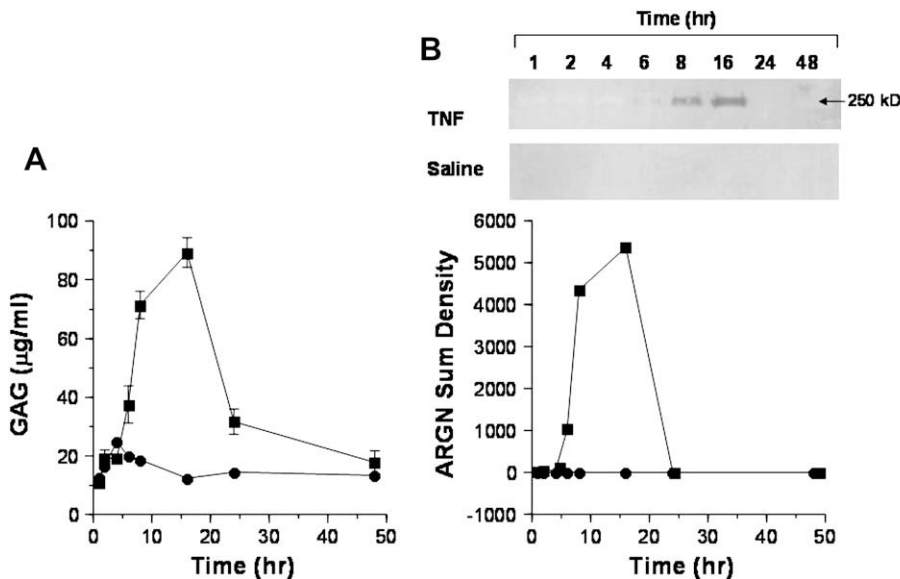|	Femoral condyle		Tibial plateau		Total score
	Lateral	Medial	Lateral	Medial	
Control ($n = 6$)	2 (1−2)	1.5 (0−2)	2 (1−2)	2 (1−2)	7 (5−8)
TNFα ($n = 7$)	0.5 (0−2)	0 (0−1)	1 (0−2)	1 (0−2)	2 (1−7)
	$P = 0.007$	$P = 0.019$	$P = 0.106$	$P = 0.160$	$P = 0.014$

Fig. 3. Time-course of TNFα-induced release of GAG and aggrecanase-generated fragments into the SF. Rats were injected IA with 10 µg TNFα [■] or with vehicle [●] and euthanized at various times (1, 2, 4, 6, 8, 16, 24 and 48 h) following challenge. (A) GAG levels in the synovial lavage fluid were determined as µg GAG per ml of synovial lavage fluid ($n = 6$ per group $\pm$ SD); (B) Lavage fluids of $n = 6$ rats were pooled by group and analyzed for the presence of the ARG neoepitope. Western blots of pooled samples at different time-points are shown (top) and the sum-density of the bands was measured by scanning densitometry (bottom).

containing fragments and thus may be useful for short term studies. In an initial study, rats were injected IA with TNFα and euthanized 6 h later. Lavage fluids were pooled and assessed by Western blot for SELE-containing fragments, formed by cleavage at the SELE[1293]/[1294]GRGT bond, as well as for ARG-containing neoepitopes [Fig. 6(A)]. The SELE antibody provided a more sensitive read-out than the ARG antibody, requiring smaller sample volumes and enabling assessment of fluids from individual animals. To
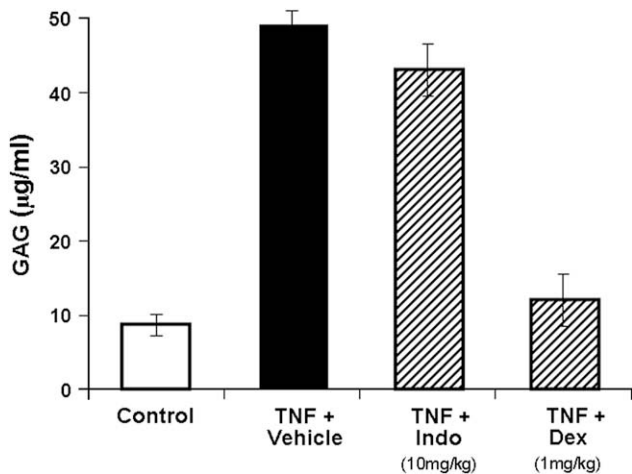

Fig. 4. Effect of indomethacin and dexamethasone on TNFα-induced proteoglycan degradation. Rats were injected IA with 10 µg TNFα or with vehicle. Immediately prior to TNFα challenge, animals were treated orally with dexamethasone (1 mg/kg), indomethacin (10 mg/kg) or with vehicle. Sixteen hours following challenge, rats were euthanized and the joints were washed with 100 µl saline. GAG levels in the synovial lavage fluid were measured, and results are presented as µg GAG per ml of synovial lavage fluid ($n = 6$ per group $\pm$ SD).

confirm that aggrecan fragments with the C-terminus SELE[1293] contained the N-terminus, ARG, and not the MMP-generated N-terminus, FFGV[22], replicate lanes were run by SDS-PAGE on pooled fluids from TNFα-challenged rats and blots were cut and assessed by SELE, ARG and FFGV Western analysis [Fig. 6(B)]. As expected, the SELE-reactive fragment was recognized by the ARG antibody, but not by the FFGV antibody, confirming that these fragments contain an N-terminus, ARG, and a C-terminus, SELE.

To assess the time-course for appearance of SELE and AGEG, another sensitive neoepitope generated by cleavage at TAQE[1584]/[1585]AGEG, a separate study was conducted where rats were injected TNFα or vehicle and euthanized at various time-points. Synovial lavage samples were analyzed for GAG levels and Western blot with the neoepitope antibodies SELE[1293] and [1585]AGEG. No or low levels of SELE- or AGEG-reactive fragments were detected in SF from vehicle controls at any of the times evaluated (not shown), whereas both neoepitopes were rapidly released into the SF in response to TNFα [Fig. 7(A)]. An AGEG-reactive fragment was detected at 120 kDa by 4 h, increasing over time through 8 h and then falling off in intensity by 16 h. A similar time-course was seen for SELE-reactive fragments. SELE fragments were detected as a doublet at ~250 kDa, the strongest band being the smaller one, which corresponds with the fragment that has [373]ARGN as the N-terminus, and therefore more likely to freely diffuse from the cartilage into the SF, as opposed to the larger band that has the intact G1 at the N-terminus[7]. Blots were quantified by scanning densitometry and plotted as a percent of maximal levels in comparison with GAG levels over time [Fig. 7(B)]. These data demonstrate that both SELE- and AGEG-containing aggrecan fragments are released over a similar time-course as GAG, suggesting that they may serve as sensitive markers of cartilage aggrecan degradation.
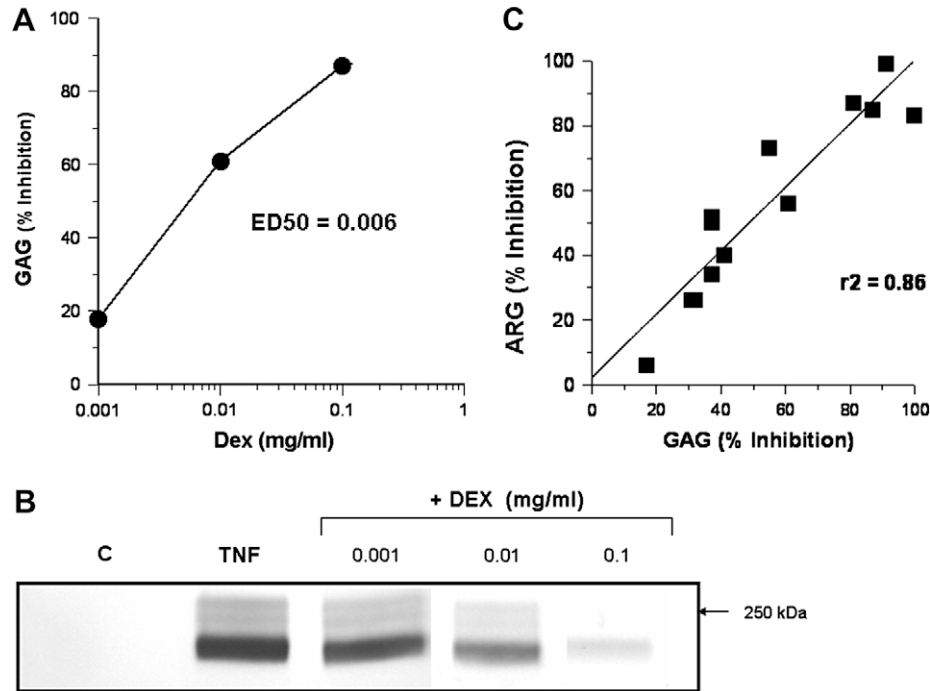
Fig. 5. Dose-related inhibition of TNFα-induced proteoglycan degradation by dexamethasone. Rats were injected IA with 10 μg TNFα or vehicle. Immediately following TNFα challenge, animals were treated by continuous IV infusion with various doses of dexamethasone (0.001, 0.01, and 0.1 mg/ml, translating to doses of 1.67, 16.7, and 167 μg/kg/h, respectively) or with vehicle for the duration of the study and euthanized 16 h following challenge. (A) GAG levels in the SF were determined on individual animals ($n = 6$/group) as μg GAG per ml lavage fluid. The mean for each Dex-treated group ($n = 6$) was then determined and % inhibition calculated compared to the mean GAG level in vehicle-treated TNFα positive control group. These % inhibition values were then plotted to determine the ED50 for inhibition by Dex; (B) Lavage fluids of $n = 6$ rats were pooled and analyzed by Western blot for the presence of the ARG neoepitope; (C) ARG Western blots from several separate studies were quantified by scanning densitometry and percent inhibition of ARG epitope generation was plotted vs percent inhibition of GAG release for each group.

EFFECT OF AN AGGRECANASE INHIBITOR

The hydroxamate aggrecanase inhibitor, compound 11, was evaluated for the ability to inhibit proteoglycan degradation in this model. Since pharmacokinetic studies showed that this compound has rapid clearance in rats, an IV bolus of 5 mg/kg was administered 1 h prior to TNFα, followed by continuous IV infusion (8650 ng/ml) until 6 h after TNFα challenge when rats were euthanized. Throughout the study, plasma levels of compound 11 were kept above 4 μM, the IC_{90} for this compound in blocking IL-1-induced bovine cartilage degradation *in vitro*. Histological staining of knee joints showed that the inhibitor prevented the loss of Safranin O staining [Fig. 8(A)], demonstrating protection from aggrecan loss. AGEG neoepitope levels in synovial lavages from individual rats were assessed by Western blot, quantitated by scanning densitometry and the mean ± s.e.m. determined [Fig. 8(B)], showing that AGEG levels were increased in TNFα-injected knee joints and reduced back to control levels in rats treated with the aggrecanase inhibitor [Fig. 8(B)].

Discussion

This work describes an acute model of cartilage degradation in the rat induced by IA TNFα injection. This cytokine induced rapid, transient cartilage proteoglycan degradation. The transient nature of the degradation is likely due to the short duration of TNFα within the joint,

as previous studies with ^{131}I-IL-1 suggest that elimination of cytokines from joint fluid occurs in less than an hour[27]. TNFα provoked primarily depletion of aggrecan from the matrix with minimal collagenolysis. In fact, cartilage degradation was mild enough to allow for repair,
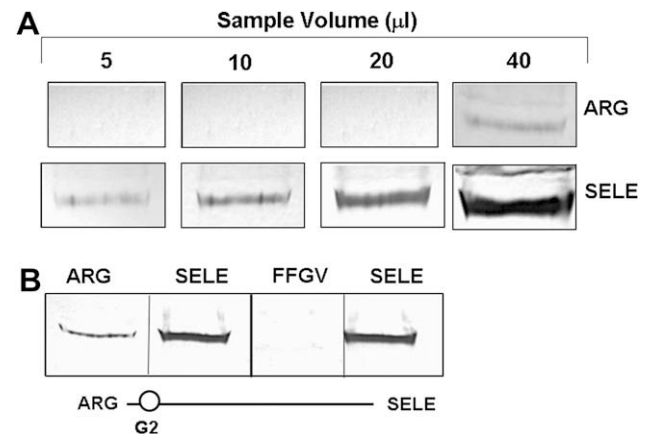

Fig. 6. Assessment of aggrecanase-generated SELE neoepitope in synovial lavage fluids. Rats were injected IA with 10 μg TNFα, euthanized 6 h following challenge and lavage samples pooled. (A) Various volumes of lavage fluid were assessed for SELE and ARG-containing neoepitopes; (B) Replicate lanes were run by SDS-PAGE and then blots cut and assessed by SELE, ARG and FFGV neoepitope Western analysis.
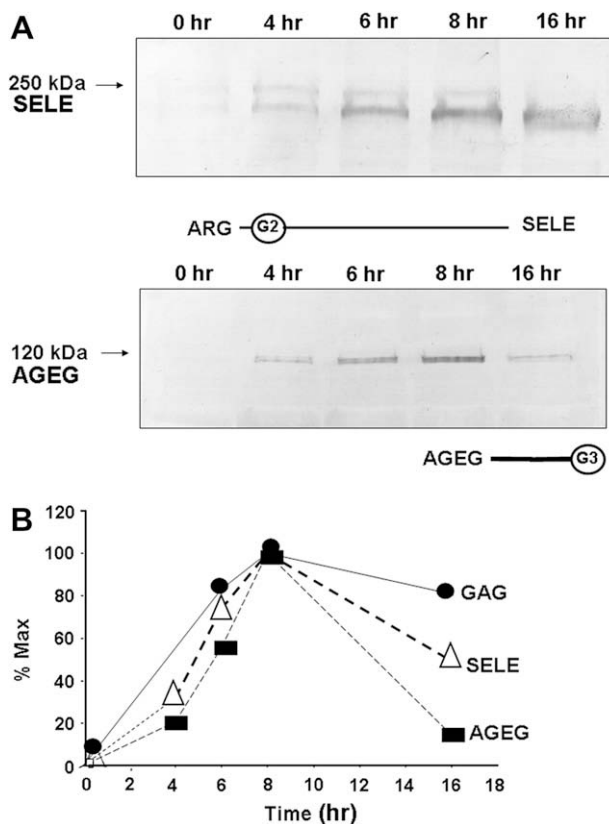
Fig. 7. Time-course of TNFα-induced release of aggrecan neoepitopes, SELE and AGEG. Rats were injected IA with 10 μg TNFα and groups of rats were euthanized at various times following challenge. (A). Lavage fluids of $n = 6$ rats were pooled and analyzed for the presence of the SELE and AGEG neoepitopes by Western blot. (B) GAG levels in of the SF lavages were measured and AGEG and SELE blots were quantified by scanning densitometry. Data are plotted as percent of maximal level vs time following TNFα challenge.

rats[37-39] and consistently resulted in cartilage catabolism. IA injection of TNFα has been much less studied[34]. A recent paper described that both IL-1β and TNFα elicited responses in the rat knee, including soft tissue inflammation and cartilage matrix loss, but only IL-1 drove skeletal resorption. Overall, IL-1β appeared to be more potent than TNFα in inducing these effects[39]. However, in developing an in vivo tool for rapid screening of chondroprotective drugs, a model characterized by a rapid onset, but mild and reversible cartilage degradation, as is observed with TNFα, offers a significant advantage.

Ideally, an in vivo model for screening pharmacological modulation of cartilage degradation should use a biomarker in a readily accessible fluid, such as blood or SF, as an endpoint read-out in order to avoid the long delay required for histological processing. The current work demonstrates that GAG levels in the SF provide a good indicator of proteoglycan loss from cartilage. However, the overall amount of GAG released into the SF cannot be determined from these levels measured at discrete time-points due to the dynamic nature of GAG appearance and loss from the fluid over the course of the study. A seminal study by Dingle and co-workers describes the IA injection of partially purified catabolin/IL-1 in rabbits and demonstrated subsequent loss of GAG from the cartilage and concomitant increase in SF GAG[27]. Our data not only confirm these early studies in another species but extend this work by demonstrating that GAG levels in the SF correspond with the appearance of aggrecan fragments cleaved specifically at the aggrecanase sites that can be used to monitor proteoglycan degradation and loss from the cartilage matrix, as well as efficacy of inhibitors in preventing this degradation.

A major finding was that IA injection of TNFα in rats results in the generation of aggrecan fragments formed by cleavage at the Glu^{373}/Ala^{374} aggrecanase site. Aggrecan fragments with the N-terminus ARGS (ARGN in rat aggrecan) have also been detected in the SF of patients with OA, inflammatory joint disease and joint injury[9,40,41]. Evaluation of SFs in the rat over a 48-h period following IA injection with TNFα indicated that there is a coordinate increase in levels of ARGN-containing fragments formed by specific cleavage at the aggrecanase site and proteoglycan catabolites as monitored by GAG levels, which correspond with proteoglycan loss from the cartilage.

Experiments with inhibitors demonstrate the potential utility of the TNFα rat model as an in vivo screening tool. The aggrecanase inhibitor study demonstrated the ability of the compound to block cartilage aggrecan depletion as assessed by Safranin O staining, and the release of aggrecan fragments into the SF, whereas a broad spectrum nanomolar MMP-inhibitor that does not inhibit ADAMTS-4/ADAMTS-5, XS309 (described in Ref. 9), was ineffective in the model, at plasma concentrations of up to 30 μM (Arner and Tortorella, unpublished data). In addition, this study demonstrated the utility of SF AGEG neoepitope levels as a rapid biomarker read-out of inhibitor efficacy. The model also differentiated standard arthritis drugs, which are known to reach the joint and provide anti-inflammatory effects, for their ability to inhibit TNFα-induced cartilage degradation. The NSAID, indomethacin, whose anti-inflammatory mechanism involves inhibition of prostaglandin production, was ineffective in blocking cartilage degradation in this model. In contrast, dexamethasone, which inhibits TNFα signaling through TNFR1 by affecting the regulation of mitogen-activated protein (MAP) kinases[42], caused a dose-related inhibition of proteoglycan release into the SF that showed a strong correlation with inhibition of the aggrecanase-

with aggrecan restored toward control levels in the tibial cartilage within 72 h after TNFα challenge. This is similar to the events observed after IA administration of IL-1 in rabbit knees, where recovery of proteoglycans occurred after the synthesis-inhibitory effects of IL-1 had worn off[28,29]. Recently, it was elegantly demonstrated that cartilage degradation in bovine cartilage explants is fully reversible in the presence of aggrecanase but not MMP activity[30]. Interestingly, recovery of proteoglycans after IA TNFα challenge was seen first in the tibial plateau (where initial depletion was least severe) before recovery in the femoral condyles (where initial depletion was earliest and most severe). This appears to be concordant with findings using delayed Gadolinium-Enhanced Magnetic Resonance Imaging of Cartilage (dGEMRIC) to study repair of cartilage explants, in which cartilage regions most severely depleted by IL-1 showed negligible GAG accumulation 3 weeks later, whereas those regions affected the least by IL-1 showed the greatest recovery[31]. Extending dGEMRIC studies to a reversible in vivo model of aggrecan depletion such as the one described here may be valuable for in vivo study of cartilage repair processes.

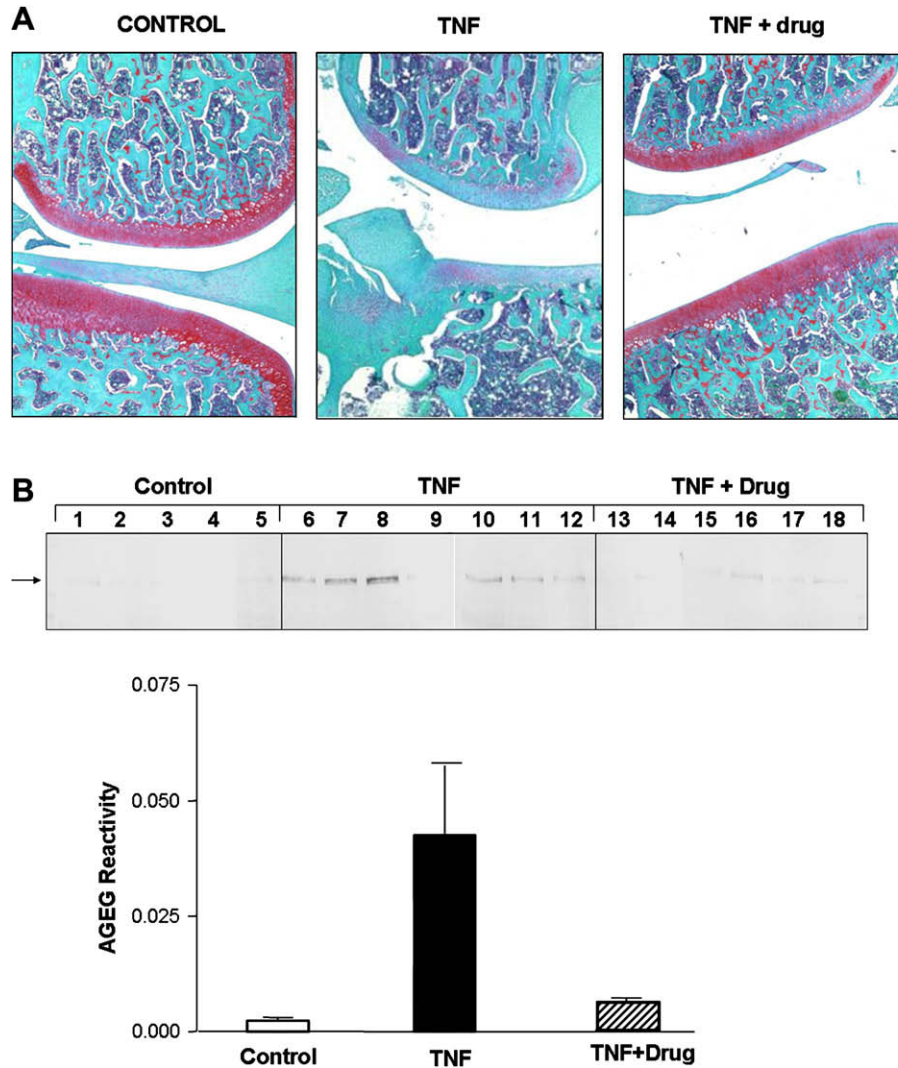IA administration of IL-1 has been extensively studied in several species, including rabbits[28,29,32-35], mice[36], and

Fig. 8. Inhibition of TNFα-induced aggrecan degradation with an aggrecanase inhibitor. Rats were injected IA with vehicle or 10 μg of TNFα, with or without continuous IV infusion of an aggrecanase inhibitor. At 6 h following challenge, rats were euthanized, synovial lavage fluids collected and knee joints taken for histology. (A) Knee joints were stained with Safranin O. Shown is the median representative of $n = 5$ for control, $n = 7$ for TNFα, and $n = 6$ for TNF + drug groups; (B) synovial lavage fluids of individual rats were analyzed for the presence of aggrecan fragments with the new N-terminus AGEG. Western blots (shown on top) were quantified by scanning densitometry and data are presented as mean $\pm$ s.e.m. of antibody reactivity.

generated neoepitope, ARGN. It should, however, be noted that, due to the specific stimulus being used (i.e., TNFα), there are obvious limitations to the predictability of this model for compound efficacy in a chronic model of OA. For example, we have found that in the rat medial meniscal tear (MMT) model[43] an aggrecanase inhibitor is very effective, whereas dexamethasone is totally ineffective (unpublished data), which may suggest that the MMT model is not TNFα-driven. Thus, compounds found to be efficacious in the acute TNFα model would ultimately need to be tested in a chronic disease model. Nevertheless, the TNFα rat model provides a rapid upstream screen for prioritizing pharmacological agents for testing in these more time- and labor-intensive models.

In summary, IA administration of TNFα in rat knee joints results in rapid, reversible aggrecan depletion from the cartilage. The fast onset of cartilage degradation and the ease of read-out in the SF *via* a biomarker that is specific for aggrecanase activity and correlates with cartilage proteoglycan loss enables rapid assessment of *in vivo* cartilage catabolism and may provide insight into potential inhibitor mechanism of action. In addition, the reversibility of aggrecan depletion may permit the use of this model for the study of cartilage repair as well as degradation.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

References

1. Mankin HJ, Lippiello L. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. J Bone Joint Surg Am 1970;52:424–34.

2. Burton-Wurster N, Todhunter RJ, Lust G. Animal models of osteoarthritis. In: Woessner JF, Howell DS, Eds. Joint Cartilage Degradation 1993;347–84.

3. Pratta W, Yao W, Decicco C, Tortorella M, Liu R, Copeland R, et al. Aggrecan protects cartilage collagen from proteolytic cleavage. J Biol Chem 2003;278:45539–45.

4. Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, et al. Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 1999;284:1664–6.

5. Abbaszade I, Liu RQ, Yang F, Rosenfeld SA, Ross OH, Link JR, et al. Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. J Biol Chem 1999;274:23443–50.

6. Curtis CL, Hughes CE, Flannery CR, Little CB, Harwood JL, Caterson B. n-3 Fatty acids specifically modulate catabolic factors involved in articular cartilage degradation. J Biol Chem 2000;275:721–4.

7. Tortorella MD, Malfait AM, Decicco C, Arner E. The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. Osteoarthritis Cartilage 2001;9:539–52.

8. Little CB, Hughes CE, Curtis CL, Jones SA, Caterson B, Flannery CR. Cyclosporin A inhibition of aggrecanase-mediated proteoglycan catabolism in articular cartilage. Arthritis Rheum 2002;46:124–9.

9. Malfait AM, Liu R-Q, Ijiri K, Komiya S, Tortorella M. Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. J Biol Chem 2002;277:22201–8.

10. Tortorella MD, Malfait AM. Will the real aggrecanase(s) step up: evaluating the criteria that define aggrecanase activity in osteoarthritis. Curr Pharm Biotechnol 2008;9:16–23.

11. Glasson SS, Askew R, Sheppard B, Carito BA, Blanchet T, Ma HL, et al. Characterization of and osteoarthritis susceptibility in ADAMTS-4-knockout mice. Arthritis Rheum 2004;50:2547–58.

12. Glasson S, Askew R, Sheppard B, Carito B, Blanchet T, Ma H, et al. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 2005;434:644–8.

13. Stanton H, Rogerson FM, East CJ, Golub SB, Lawlor KE, Meeker CT, et al. ADAMTS5 is the major aggrecanase in mouse cartilage in vivo and in vitro. Nature 2005;434:648–52.

14. Majumdar MK, Askew R, Schelling S, Stedman N, Blanchet T, Hopkins B, et al. Double-knockout of ADAMTS-4 and ADAMTS-5 in mice results in physiologically normal animals and prevents the progression of osteoarthritis. Arthritis Rheum 2007;56:3670–4.

15. Botter SM, Glasson SS, Hopkins B, Clockaerts S, van Leeuwen JP, Weinans H, et al. ADAMTS5-/- mice have less subchondral bone changes after induction of osteoarthritis through surgical instability: implications for a link between cartilage and subchondral bone changes. Osteoarthritis Cartilage 2008. doi:10.1016/j.joca.2008.09.018.

16. Song RH, Tortorella MD, Malfait AM, Yang Z, Alston JT, Arner EC, et al. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis Rheum 2007;56:575–85.

17. Bendele AM. Animal models of osteoarthritis. J Musculoskelet Neuronal Interact 2001;1:363–76.

18. Ameye LG, Young MF. Animal models of osteoarthritis: lessons learned while seeking the "Holy Grail". Current Opinion Rheum 2006;18:537–47.

19. Wittwer AJ, Hills RL, Keith RH, Munie GE, Arner EC, Anglin CP, et al. Substrate-dependent inhibition kinetics of an active site-directed inhibitor of ADAMTS-4 (Aggrecanase 1). Biochemistry 2007;46:6393–401.

20. Hughes CE, Caterson B, Fosang AJ, Roughley PJ, Mort JS. Monoclonal antibodies that specifically recognize neoepitope sequences generated by 'aggrecanase' and matrix metalloproteinase cleavage of aggrecan: application to catabolism in situ and in vitro. Biochem J 1995;305:799–804.

21. Tortorella MD, Pratta M, Liu RQ, Austin J, Ross OH, Abbaszade I, et al. Sites of aggrecan cleavage by recombinant human aggrecanase-1 (ADAMTS-4). J Biol Chem 2000;275:18566–73.

22. Fosang AJ, Last K, Gardiner P, Jackson DC, Brown L. Development of a cleavage-site-specific monoclonal antibody for detecting metalloproteinase-derived aggrecan fragments in human synovial fluids. Biochem J 1995;310:337–43.

23. Yao W, Wasserman ZK, Chao M, Reddy G, Shi E, Liu RQ, et al. Design and synthesis of a series of (2r)-n(4)-hydroxy-2-(3-hydroxybenzyl)-n(1)-[(1s,2r)-2-hydroxy-2,3-dihydro-1h-inden-1ryl]butanediamide derivatives as potent, selective, and orally available aggrecanase inhibitors. J Med Chem 2001;21:3347–50.

24. Oke SL, Hurtig MB, Keates RA, Wright JR, Lumsden JH. Assessment of three variations of the 1,9-dimethylmethylene blue assay for measurement of sulfated glycosaminoglycan concentrations in equine synovial fluid. Am J Vet Res 2003;64:900–6.

25. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.

26. Tortorella MD, Liu R-Q, Burn T, Newton RC, Arner E. Characterization of human aggrecanase 2 (ADAM-TS5): substrate specificity studies in comparison with aggrecanase 1 (ADAM-TS4). Matrix Biol 2002;21:499–511.

27. Dingle JT, Page Thomas DP, King B, Bard DR. In vivo studies of articular tissue damage mediated by catabolin/interleukin 1. Ann Rheum Dis 1987;46:527–33.

28. Arner EC, Di Meo TM, Ruhl DM, Pratta MA. In vivo studies on the effects of human recombinant interleukin-1 beta on articular cartilage. Agents Actions 1989;27:254–7.

29. Page Thomas DP, King B, Stephens T, Dingle JT. In vivo studies of cartilage regeneration after damage induced by catabolin/interleukin-1. Ann Rheum Dis 1991;50:75–80.

30. Karsdal M, Madsen S, Christiansen C, Henriksen K, Fosang A, Sondergaard B. Cartilage degradation is fully reversible in the presence of aggrecanase but not matrix metalloproteinase activity. Arthritis Res Ther 2008;10 (Epub ahead of print).

31. Williams A, Oppenheimer RA, Gray ML, Burstein D. Differential recovery of glycosaminoglycan after IL-1-induced degradation of bovine articular cartilage depends on degree of degradation. Arthritis Res Ther 2003;5:R97–R105.

32. Pettipher ER, Higgs GA, Henderson B. Interleukin 1 induces leukocyte infiltration and cartilage proteoglycan degradation in the synovial joint. Proc Natl Acad Sci U S A 1986;83:8749–53.

33. Henderson B, Pettipher ER. Comparison of the in vivo inflammatory activities after intra-articular injection of natural and recombinant IL-1a and IL-1b in rabbits. Biochem Pharmacol 1988;37:4171–6.

34. O'Byrne EM, Blancuzzi V, Wilson DE, Wong M, Jeng AY. Elevated substance P and accelerated cartilage degradation in rabbit knees injected with interleukin-1 and tumor necrosis factor. Arthritis Rheum 1990;33:1023–8.

35. Lundberg C, Asberg I, Ionescu M, Reiner A, Smedegard G, Poole AR. Changes in cartilage proteoglycan aggrecan after intra-articular injection of interleukin-1 in rabbits: studies of synovial fluid and articular cartilage. Ann Rheum Dis 1996;55:525–34.

36. van Beuningen HM, Arntz OJ, van den Berg WB. In vivo effects of interleukin-1 on articular cartilage. Prolongation of proteoglycan metabolic disturbances in old mice. Arthritis Rheum 1991;34:606–15.

37. Chandrasekhar S, Harvey AK, Hrubey PS, Bendele AM. Arthritis induced by interleukin-1 is dependent on the site and frequency of intraarticular injection. Clin Immunol Immunopathol 1990;55:382–400.

38. van de Loo AA, Arntz OJ, Otterness IG, van den Berg WB. Proteoglycan loss and subsequent replenishment in articular cartilage after a mild arthritic insult by IL-1 in mice: impaired proteoglycan turnover in the recovery phase. Agents Actions 1994;41:200–8.

39. Bolon B, Campagnuolo G, Zhu L, Duryea D, Zack D, Feige U. Interleukin-1beta and tumor necrosis factor-alpha produce distinct, time-dependent patterns of acute arthritis in the rat knee. Vet Pathol 2004;41:235–43.

40. Sandy JD, Flannery CR, Neame PJ, Lohmander LS. The structure of aggrecan fragments in human synovial fluid. Evidence for the involvement in osteoarthritis of a novel protease which cleaves the Glu373–Ala374 bond of the interglobular domain. J Clin Invest 1992;89:1512–6.

41. Lohmander LS, Neame PJ, Sandy JD. The structure of aggrecan fragments in human synovial fluid. Evidence that aggrecanase mediates cartilage degradation in inflammatory joint disease, joint injury, and osteoarthritis. Arthritis Rheum 1993;36:1214–22.

42. Abraham S, Lawrence T, Kleiman A, Warden P, Medghalchi M, Tuckermann J, et al. Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. J Exp Med 2006;203:1883–9.

43. Janusz MJ, Bendele AM, Brown KK, Taiwo YO, Hsieh L, Heitmeyer SA. Induction of osteoarthritis in the rat by surgical tear of the meniscus: inhibition of joint damage by a matrix metalloproteinase inhibitor. Osteoarthritis Cartilage 2002;10:785–91.