OsteoArthritis and Cartilage (2007) **15,** 212–221

doi:10.1016/j.joca.2006.07.009

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

MMP and non-MMP-mediated release of aggrecan and its fragments from articular cartilage: a comparative study of three different aggrecan and glycosaminoglycan assays

E. U. Sumer M.Sc.†, B. C. Sondergaard M.Sc.†, J. C. Rousseau Ph.D.‡, P. D. Delmas M.D., Ph.D.‡, A. J. Fosang Ph.D.§, M. A. Karsdal Ph.D.†, C. Christiansen D.M.Sc.‖ and P. Qvist Ph.D.†*

† *Nordic Bioscience, Herlev, Denmark*

‡ *Inserm Unit 403 and Synarc, Pavillon F, Hôpital E. Herriot, Lyon, France*

§ *Department of Paediatrics, Murdoch Children's Research Institute, Royal Children's Hospital, University of Melbourne, Parkville, Australia*

‖ *CCBR, Ballerup, Denmark*

Summary

Objective: Aggrecan is the major proteoglycan in articular cartilage and is known to be degraded by various proteases, including matrix metalloproteinases (MMPs). The present study was undertaken to develop immunoassays detecting aggrecan and its fragments generated by MMP and non-MMP-mediated proteolysis.

Methods: Two immunoassays were developed: (1) the G1/G2 sandwich assay employing a monoclonal antibody (F-78) both as a capturing and a detecting antibody, and (2) the 342-G2 sandwich assay substituting the capturing antibody in the G1/G2 test with a monoclonal antibody, AF-28 recognizing the ^{342}FFGVG neo-epitope generated by MMP cleavage. These assays were compared to the commercially available glycosaminoglycan (GAG) assay.

Results: In supernatants of Oncostatin M and Tumor Necrosis Factor alpha (OSM/TNFα) stimulated explants, high levels of G1/G2 fragments and GAGs were released in the initial phase (days 2–5), followed by low levels in the intermediate (days 9–12) and late phase (days 12–21). MMP-generated fragments were detected in the late phase only. In the presence of the general MMP inhibitor GM6001, 342-G2 was not detected, whereas the G1/G2 profile remained virtually unchanged. In patients with rheumatoid arthritis (RA), the release of G1/G2 molecules was decreased (27.3%), and that of the 342-G2 fragments increased compared to healthy controls (33.3%).

Conclusion: The stimulation of bovine articular cartilage explants with OSM/TNFα released aggrecan fragments both in an MMP and non-MMP-mediated route. These immunoassays carry a potential as diagnostic tools for the quantitative assessment of the cartilage turnover in RA patients in addition to their utility in *ex vivo* explant cultures.

Key words: Aggrecan, Matrix metalloproteinases, Articular cartilage explants, Glycosaminoglycan, Arthritis.

Introduction

A central pathophysiological event in osteoarthritis (OA) and rheumatoid arthritis (RA) is the irreversible destruction of articular cartilage of the joints. Consequently, the gradual loss of cartilage in the joints leads to pain and functional impairment[1].

In articular cartilage, chondrocytes synthesize extracellular matrix (ECM) molecules such as collagens, proteoglycans and hyaluronic acid. These components are anchored in an organized and complex structure capable of attracting and retaining large quantities of water, and thereby acquiring the ability to resist compressive deformation during mechanical loading[2]. In RA, the chondrocytes concomitantly with the synovium[3,4] are responsible for the degradation of the ECM by producing a wide variety of proteases that can degrade type II collagen, aggrecan and other components of the cartilage matrix[5–7].

To facilitate the study of chondrocyte-mediated cartilage metabolism, several *in vitro* and *ex vivo* model systems have been developed. In particular, the culturing of articular cartilage explants offers the advantage of having the chondrocytes embedded in their natural matrix, allowing the cells to interact within the complex environment of the cartilage[8].

The degradation of type II collagen has been studied in *ex vivo* cultures of bovine articular explants[9,10]. The release of collagen fragments into the supernatant has been quantitatively assessed by immunoassays[11], or alternatively, by quantifying hydroxyproline[12].

Likewise, cartilage erosion in explant cultures has been quantified by monitoring the level of glycosaminoglycans (GAGs) released into the supernatant[13–17], or by detecting various aggrecan fragments[7,14–19]. Previous identification and characterization of aggrecan neo-epitopes generated

*Address correspondence and reprint requests to: Per Qvist, Ph.D., Nordic Bioscience A/S, Herlev Hovedgade 207, DK-2730 Herlev, Denmark. Tel: 45-44-54-77-04; Fax: 45-44-54-88-88; E-mail: eus@nordicbioscience.com, bcs@nordicbioscience.com, rousseau@lyon.inserm.fr, delmas@lyon.inserm.fr, amanda.fosang@mcri.edu.au, mk@nordicbioscience.com, cc@ccbr.dk, pq@nordicbioscience.com

Received 7 April 2006; revision accepted 23 July 2006.

through the action of matrix metalloproteinases (MMPs) and ADAMTs (A Disintegrin and Metalloproteinase with Thrombospondin motifs) have been conducted mostly by Western blotting techniques after stimulation with different pro-inflammatory agents[7,14–19]. Immunoassays detecting neo-epitopes of aggrecan formed by the action of MMPs and ADAMTs have been reported by Fosang *et al.*[20] and Pratta and co-workers[21]. Though there are a number of ADAMTs and MMP cleavage sites in aggrecan, the major MMP and ADAMTs cleavage sites are located between IPE-N^{341}–^{342}FFGV and TEGE^{373}–^{374}ARGS in the interglobular domain (IGD), respectively. However, these studies have not systematically delineated the MMP-mediated and non-MMP-mediated release of aggrecan into the supernatant of cartilage explant cultures by enzyme-linked immunosorbent assay (ELISA).

Therefore, the present study was undertaken to develop corresponding immunoassays allowing the investigation of MMP-mediated and non-MMP-mediated release of aggrecan and its fragments, and to use these in the investigation of cartilage degradation in bovine articular cartilage explants and in patients with RA.

Materials and methods

CLINICAL DATA

The clinical data were evaluated by randomly selecting 20 RA patients (age of 59 ± 4.8 years) from a 56 week study, who all fulfilled the American College of Rheumatology (ACR) criteria for RA disease revised in 1987[22]. All patients were female and had early RA <2 years duration (median duration 4 months). Patients underwent drug treatment either with leflunomide, methotrexate, sulfasalazine, trolovol alone, or in combination with low dosage of corticosteroid (5–10 mg/day prednisolone) for 2 years before they were evaluated in the study. Patients with renal insufficiency were excluded. A subset of 57 healthy age-matched women (age of 57 ± 2.7) were used as controls for patients with RA.

Blood was collected in tubes from fasting individuals in the morning before 10 AM, and allowed to clot at room temperature (RT) for at least 10 min. Then the collected blood was centrifuged for 10 min at 1000*g*. The serum was isolated from the clot, and stored at -70°C, before assaying them in two developed aggrecan assays (see below). Disease Activity Score (DAS) was 5.2 ± 1.1 (mean $\pm$ SD) in the RA patients. DAS is a validated index that includes a count of 28 joints assessed for swelling and tenderness, a measure of an acute-phase reactant and a measure of global well-being[23].

Anteroposterior radiographs of the hand, wrist and feet were obtained from baseline, after 28 weeks, and at the end of the trial. The radiographs were evaluated by two independent persons who were blinded to the treatment but were aware of the chronological sequence of the radiographs. Radiographs were scored according to the modified Sharp/van der Heide scoring methods[24]. The Sharp score was 135 ± 87 (mean $\pm$ SD) in the RA patients.

REAGENTS

Dulbecco's modified Eagle's medium (DMEM) (cat. number 22320-022), Roswell Park Memorial Institute (RPMI) 1640 medium (cat. number 52400-025), Hypoxanthine and Thymidine (HT) supplement 100$\times$ (cat. number 11067-030), Hypoxanthine Aminopterin Thymidine (HAT) supplement 50$\times$ (cat. number 21060-017), penicillin 10,000 U/ml and streptomycin 10,000 μg/ml (cat. number DE 17-602E) and Fetal Calf Serum (FCS) (cat. number 10082-147) were all purchased from Invitrogen, DK. Delfia Europium (EU)-N1 labeled anti-mouse antibody (AD0124), assay buffer (50 mM Tris–HCl, 0.9% NaCl, 0.5% Bovine Serum Albumin (BSA), 0.1% Tween, 20 μM ethylenediaminetetraacetic acid (EDTA), pH 7.8) (cat. number 1244-111) and enhancement solution (acidic chelating detergent) (cat. number 4001-0010) were all from Perkin Elmer. Hybridoma cloning factor (contains various growth factors) (cat. number S05-015) was bought from PAA laboratory GmBH. The MMP inhibitor GM6001 2.5 mM (CC1000), synthetic IGD of aggrecan 500 μg/ml (CC1890) and chicken neurocan 10 μg (AG270) and Freunds incomplete adjuvants 50 ml (AR002) were all from Chemicon, USA. Tumor Necrosis Factor alpha (TNFα) 10 μg/ml (cat. number 210-TA) and MMP-13 (cat. number 511-MM) were bought from R and D systems. Maxisorp plates were purchased from Nunc. (cat. number 439454), while streptavidin coated plates were bought from Roche (cat. number 1207733). Purified bovine aggrecan 1 mg (A-1960), Chondroitinase ABC Lyase 0.3–3 U/mg solid (C2905), oncostatin M (OSM) 10 μg/ml (O9635-10UG), Pepstatin A (P5318) 10 mg, *p*-aminophenylmercuric acetate (APMA) 5 g (A-9563), MMP-2 200 μg/ml (M-7942), MMP-9 200 μg/ml (M-4809) and gelatine 100 g from bovine skin (G9382) were all from Sigma Aldrich, DK.

DEVELOPMENT OF MONOCLONAL ANTIBODIES TO THE G1/G2 DOMAIN OF AGGRECAN

Eight to 10-week-old Balbc/A mice were immunized subcutaneously every second week for 2 months and subsequently monthly with 50 μg per dose of purified bovine intact aggrecan emulsified in an equal volume of Freunds incomplete adjuvant. Serum from immunized mice was collected, and the titer of anti-aggrecan antibodies assessed by fluorescence immunoassay. This was done by coating maxisorp plates overnight (ON) at 4°C with 100 μl chondroitinase-treated aggrecan diluted to 25 ng/ml in coating buffer (10.6 mM Na_2CO_3, 39.3 mM $NaHCO_3$, 3.1 mM NaN_3, pH 9.6). The plates were washed five times with washing buffer (0.15 M NaCl, 0.05% (v/v) Tween 20). Subsequently, 100 μl of mice serum serial diluted in Phosphate Buffered Solution with Bovine Serum Albumine and Tween (PBS-BTB) was added to the wells, and the plate was incubated for 1 h at 4°C with shaking at 300 rpm. After washing as described above, 100 μl of 0.5 μg/ml Delfia EU-labeled secondary anti-mouse antibody diluted in assay buffer was added to the wells, and the plate was incubated for 1 h at 4°C with shaking. After washing the plate five times, 150 μl enhancement solution (acidic chelating substrate solution for determination of formed EU^{3+}/Sm^{3+} ions in solution in fluorescence ELISA, Perkin Elmer) was added, and after incubating the plate for 15 min at RT with shaking, the fluorescence was measured with a wallas victor^2 1420 multilabeled counter.

Mice with high aggrecan antibody titers were intra-peritoneally injected with 50 μg of purified bovine aggrecan 3 days before fusion. Fusion was performed after standard procedures with ECACC X+4P P3X63.Ag8-653 myeloma cells as previously described[25].

Supernatants from hybridoma cells were screened for the presence of anti-aggrecan antibodies as described above. Hybridomas from wells containing anti-aggrecan antibodies were selected and cloned twice by limiting dilution. Subsequently, the monoclonal antibodies were evaluated in a series of immunoassays. Briefly, antibodies recognizing purified porcine G1/G2[26] (material characterized in Ref. [26]), but

not synthetic IGD, were subsequently tested for applicability in a sandwich construction with the monoclonal antibody AF-28, recognizing the N-terminal neo-epitope ^{342}FFGVG (see below). One monoclonal antibody, i.e., F-78, was selected for further development.

SPECIFICITY INVESTIGATIONS OF F-78

Cross-reactivity to other proteoglycans was investigated by competition G1/G2 ELISA. Shortly, 100 µl 1000 ng/ml biotinylated bovine aggrecan was incubated on streptavidin coated plates for 1 h at 20°C with shaking (300 rpm). After washing the plates five times, 50 µl 10,000 ng/ml of aggrecan, biglycan, BSA, decorin or neurocan were added to the wells at the same time as 100 µl 300 ng/ml peroxidase (POD)-labeled F-78. After incubating the plates for 1 h at 20°C with shaking (300 rpm), they were washed five times, and 100 µl of 3,3′,5,5′-tetramethylbenzidine solution (TMB) substrate was added, and the plates were incubated for 15 min at 20°C with shaking in the dark. Finally, 100 µl of 0.18 M H_2SO_4 was added to the wells, and the absorbance was measured at 450 nm.

To further characterize the specificity of F-78, the G1/G2 sandwich assay (see below) was also run with purified porcine G1[27] (material described in Ref. [26]) at a concentration of 1000 ng/ml. As a negative control, BSA at the same concentration was used.

BOVINE ARTICULAR CARTILAGE EXPLANTS

Bovine articular cartilage was obtained from the stifle joints of a 15-month-old bovine from the local slaughtery. Pieces of cartilage (14 ± 2 mg) were placed in 96-well plates and incubated for 18 days at 37°C with 5% CO_2 and shaking (50 rpm). Serum-free DMEM was used. Explants were incubated with (1) medium alone, (2) 10 ng/ml OSM and 20 ng/ml TNFα of the pro-inflammatory cytokines in combination[28], or (3) Oncostatin M and Tumor Necrosis Factor alpha (OSM/TNFα) plus 10 µM (physiologically relevant concentration)[18] of the MMP inhibitor GM6001[29,30]. As a negative control, cartilage was metabolically inactivated (MI) by freezing in liquid N_2, and thawed at 37°C in waterbath for three repeated freeze–thaw cycles. The explant culture medium was replaced every second to third day for 18 days, and six independent replicates were set up of each condition. The corresponding supernatants were pooled, and stored at −20°C until further analysis. Concentrations reported are subtracted from the negative control.

DETECTION OF AGGRECAN FRAGMENTS CONTAINING G1/G2

Monoclonal antibody F-78 was used in a sandwich construction as both a capturing and a detecting antibody, for the quantification of aggrecan or its fragments containing G1 and/or G2 molecules. Streptavidin coated plates were incubated for 1 h at 20°C with 100 µl of biotinylated F-78 diluted to 1800 ng/ml in PBS-BTB buffer. Plates were incubated with shaking (300 rpm), and then washed five times with washing buffer (0.15 mol/l NaCl, 0.05% (v/v) Tween 20). Subsequently 100 µl standards (purified bovine aggrecan 0.0625–1 ng/ml), kit control (purified bovine aggrecan 0.5 ng/ml) or unknown samples (explant supernatants or serum samples) were added, and the plates were incubated for 1 h at 20°C with shaking. After the incubation period, the plates were washed five times as described above, and 100 µl of POD-labeled F-78 antibody diluted to 2000 ng/ml in PBS-BTB was added. After incubating for 1 h at 20°C with shaking, the plates were washed five times and 100 µl of TMB substrate was added, and the plates were incubated for 15 min at 20°C with shaking in the dark. Finally, 100 µl of 0.18 M H_2SO_4 was added to the wells, and the absorbance was measured at 450 nm.

DETECTION OF AGGRECAN FRAGMENTS CONTAINING 342-G2

Monoclonal antibody AF-28 recognizing the N-terminal neo-epitope generated by proteolytic cleavage of the amino acid sequence DIPEN^{341}–^{342}FFGVG localized in the IGD has previously been described[20]. The 342-G2 test was very similar to the G1/G2 test described above except that the capturing antibody, i.e., biotinylated F-78, was substituted with biotinylated AF-28 at a concentration of 1500 ng/ml in PBS-BTB. In addition, MMP-13 digested purified porcine laryngeal aggrecan diluted from 46–3000 ng/ml in PBS-BTB was used as standards. Furthermore, run samples were kit control (FCS 1500 ng/ml) or unknown samples (explant supernatants or serum samples).

For the digestion of porcine laryngeal aggrecan 50 µl of 5 mg/ml aggrecan was digested with 25 µl of 400 nM MMP-13 (1 mM APMA activated ON at 37°C) with the proteinase inhibitors 7 µl of 0.1 mg/ml Pepstatin, and 1 µl of 20 mM E-64 in 417 µl MMP buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 0.15 M NaCl, 0.05% Brij35). Digestion was done ON at 37°C with gentle shaking.

TECHNICAL PERFORMANCE

Briefly, for the determination of intra-assay, samples were run in 24 replicates, and their average was calculated on the basis of this. For determining the inter-assay, each sample was run as double replicates at 10 independent days, and the average was calculated.

DETECTION OF GAGs

For detection of sulfated GAGs, the quantitative dye-binding assay for *in vitro* analysis of GAG release was used according to the manufacturer's instructions (Wieslab, Sweden). Briefly, 50 µl of samples (explant supernatants), standards (chondroitin sulfate-6 12.5–400 µg/ml), blank (water), and kit control (cartilage extract diluted in water) were pipetted into a vial and 50 µl 8 M Guanidine–HCl was added. The vials were incubated for 15 min at RT. After incubation, 50 µl SAT solution (0.3% H_2SO_4 and 0.75% Triton X-100) was added into each vial and they were incubated for 15 min at RT. 750 µl Alcian Blue (0.3%) working solution (0.1% H_2SO_4 and 0.4 M GuHCl) was added, the contents were mixed, and incubated for 15 min at RT. After incubation, the pellet was isolated by centrifugation for 15 min at 12,000*g*. MSO solution (40% dimethylsulphoxide and 0.05 M $MgCl_2$) (500 µl) was added, and mixing was done thoroughly on a shaker for 15 min at RT. This was followed by centrifugation for 15 min at 12,000*g*, and the supernatant was removed. Gu–Prop solution (4 M GuHCl, 33% 1-propanol and 0.25% Triton X-100) (500 µl) was added to the pellets and the vials were shaken for 15 min at RT. The absorbance was read at 620 nm.

PROTEOLYTIC PROCESSING OF AGGRECAN AND ITS FRAGMENTS RELEASED INTO THE SUPERNATANT

All reactions were carried out in 0.5 ml of MMP buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$, 0.15 M NaCl,

0.05% Brij35). OSM/TNFα treated explant supernatants from day 5 and day 18 (see the section on Bovine articular cartilage explants) were selected for sensitivity to further proteolytic processing with MMPs. Each supernatant was incubated with either MMP-2 or MMP-9 activated by incubation with 1 mM APMA ON at 37°C. After activation, 10 μl of enzyme suspension was added to 50 μl of the supernatant in a final concentration of 100 nM MMP-2 or MMP-9 and 440 μl buffer, and the solutions were incubated for 24 h at 37°C under gentle agitation. After incubation, the reaction was stopped by adding 20 μl of 250 μM GM6001 and stored frozen until further analysis.

ZYMOGRAPHY

MMP expression and activity was determined by gelatinase zymography using 0.5 mg/ml of gelatine as a substrate in 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. A volume of 5 μl of culture supernatant was loaded on the zymography gels, and the proteins were separated. After electrophoresis, the gels were washed three times with 2.5% Triton X-100 in water and then incubated ON at 37°C in 0.1% Triton X-100, 5 mM $CaCl_2$, 1 mM $ZnCl_2$, 3 mM NaN_3, 50 mM Tris pH 7.4 in a closed container. Gels were stained for 30 min with 0.25% Coomassie R-250 in 10% acetic acid and 45% methanol and destained for 30 min with 20% acetic acid, 20% methanol, 17% ethanol, and 0.6% diethylether. Gels were dried and scanned for documentation.

HISTOLOGY

The bovine articular cartilage explants were stimulated with OSM/TNFα and fixed in 3.7% formaldehyde in PBS pH 7.4, paraffin embedded, and subsequently sectioned into 5 μm sections. The proteoglycans were stained with toluidine blue after deparaffinization and rehydration. The sections were counterstained with Erlich's hematoxylin. Digital histographs were taken using an Olympus BX-60 microscope and an Olympus C5050 zoom camera.

Results

DEVELOPMENT OF IMMUNOASSAYS FOR AGGRECAN

Monoclonal antibody F-78 was raised against intact bovine aggrecan, and its specificity was tested by a competition G1/G2 ELISA. The antibody was particularly specific to aggrecan, and showed no appreciable cross-reactivity to biglycan and decorin, while F-78 demonstrated low reactivity to neurocan [Fig. 1(A)]. To further characterize its specificity, it was incorporated into a sandwich immunoassay. Reactivity of purified porcine G1 domain was observed in the sandwich ELISA [Fig. 1(B)].

For detection of MMP-generated aggrecan fragments, a sandwich assay with AF-28 as capturing antibody was developed. Reactivity of purified MMP digested porcine aggrecan was detected [Fig. 1(C)].

The technical performance of both the G1/G2 and 342-G2 sandwich tests was investigated (Table I). Both tests had intra- and inter-assay variations in the range 5.8–8.9%, and the linearity was between 103.9–116.9% and 95.7–98.1% for the G1/G2 and the 342-G2 tests respectively. These two tests [Fig. 2(A and B)] were compared to the test for total GAGs.

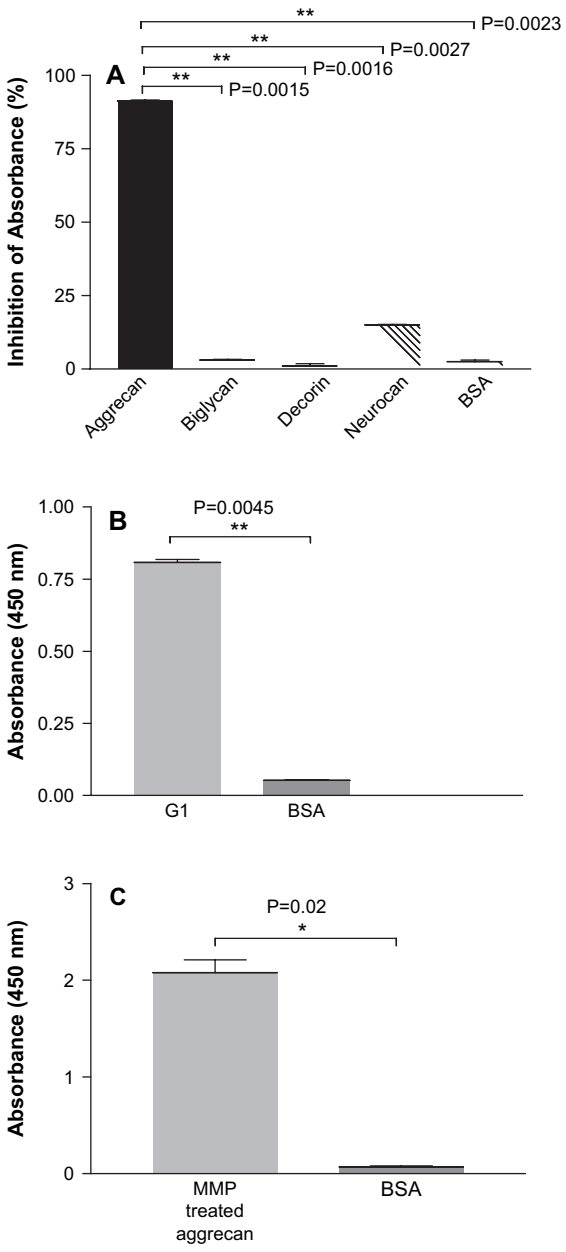

Fig. 1. (A) To investigate the specificity of F-78, intact purified bovine aggrecan, chicken neurocan, BSA as a negative control, bovine biglycan and bovine decorin were run in the G1/G2 competition assay at a concentration of 10,000 ng/ml. The values are mean + S.E.M.S, and the asterisks indicate significant differences. For the statistical analysis, unpaired *t* test with Welch's correction was used with one-tailed *P* values. (B) To investigate the specificity of F-78, purified porcine G1 and BSA as a negative control were run in the G1/G2 sandwich assay at a concentration of 1000 ng/ml. The values are mean + S.E.M.S, and the asterisks indicate significant differences. For the statistical analysis, unpaired *t* test with Welch's correction was used with one-tailed *P* values. (C) To investigate the specificity of F-78, purified MMP digested porcine aggrecan was run in the 342-G2 sandwich assay at a concentration of 1000 ng/ml, and as a negative control BSA was used. The values are mean + S.E.M.S, and the asterisks indicate significant differences. For the statistical analysis, unpaired *t* test with Welch's correction was used with one-tailed *P* values.

Table I
Technical performance of the G1/G2 and the 342-G2 tests

	Total aggrecan turnover	Aggrecan degradation
	G1/G2 assay	342-G2 assay
Measuring range	0.063–1.000 ng/ml	46–3000 ng/ml
Lower Detection Limit (LDL)	0.010 ng/ml	10 ng/ml
Intra-assay ($n=24$) (mean; coefficient of variation)	0.781 ng/ml; 7.1%	995 ng/ml; 7.5%
Inter-assay ($n=10$) (mean; coefficient of variation)	0.22 ng/ml; 8.9%	816 ng/ml; 5.8%
Dilution recovery (mean; range)	110.4%; 103.9–116.9%	96.8%; 95.7–98.1%

RELEASE OF AGGRECAN AND ITS FRAGMENTS FROM ARTICULAR CARTILAGE EXPLANTS

Explants, all in the range of 12–16 mg, from bovine articular cartilage were isolated, and conditioned medium from pooled supernatants (low variability) stimulated with OSM/TNFα demonstrated an increase in the release of 342-G2 fragments in the late phase after day 12 (Fig. 3). The elevated release in the late phase was completely abrogated in the presence of the MMP inhibitor GM6001. Under the same experimental conditions, the release of G1/G2 had a different profile with elevated release in the early phase approaching background levels in the mid and late phase. The G1/G2 profile was not diminished in the presence of MMP inhibitor (Fig. 3). However, total GAG release into the supernatant had a profile similar to the G1/G2 profile, with elevation in the initial phase only (Fig. 4). Supernatants obtained from MI cartilage explants, or explants kept in medium without catabolic cytokines, contained less than around 10 μg/ml of aggrecan or aggrecan fragments throughout the whole period (data not shown).

EXPRESSION AND ACTIVITY OF MMP-2 AND MMP-9 INDUCED BY TNFα AND OSM STIMULATION OF BOVINE ARTICULAR CARTILAGE EXPLANTS

We investigated the expression and activity of gelatinases by zymography of explant supernatants stimulated

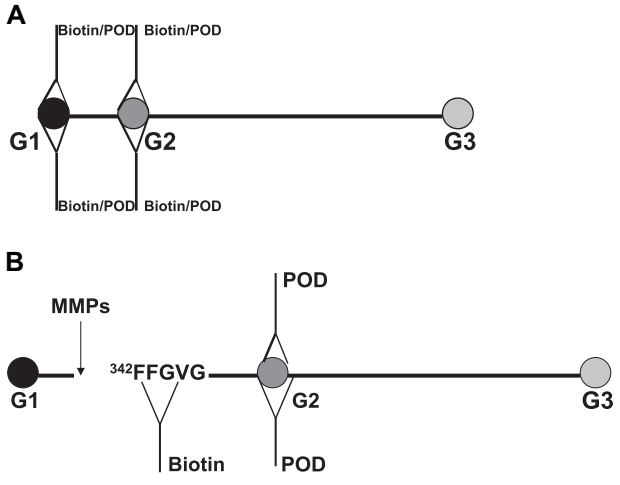

Fig. 2. (A) The G1/G2 assay detecting intact aggrecan and aggrecan fragments carrying the G1 and/or G2 domain, (B) 342-G2 assay detecting aggrecan fragments carrying both the ^{342}FFGVG neo-epitope and the G2 globular domain.

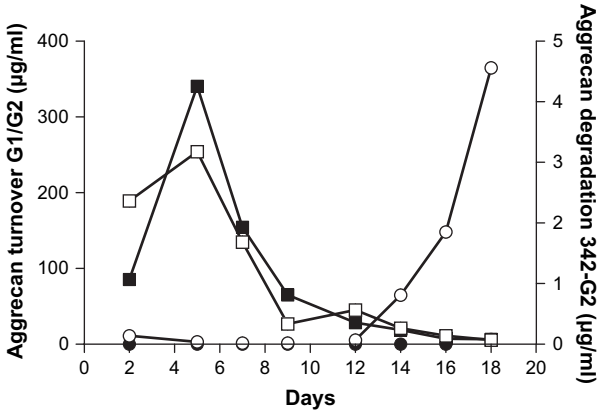

Fig. 3. Bovine articular cartilage explants were stimulated with the cytokines OSM/TNFα and supernatants were measured for the presence of aggrecan and aggrecan fragments by immunoassays. G1/G2 assay with (-■-) and without (-□-) the general MMP inhibitor GM6001. 342-G2 assay with (-●-) and without (-○-) GM6001.

with OSM/TNFα (Fig. 5). While the pro-enzymes of MMP-2 and MMP-9 were expressed at early days in the supernatants of OSM/TNFα stimulated bovine cartilage explants, the active enzymes were detected from day 14 (MMP-2) and day 12 (MMP-9) and onwards. As control for MMP activity, the gelatine zymography was incubated with GM6001, which completely blocked gelatinase activity (data not shown). The molecular weight marker showed the bands corresponding to the weights of pro MMP-9 92 kDa, active 86 kDa and pro MMP-2 72 kDa and active 66 kDa.

FURTHER PROTEOLYTIC PROCESSING OF AGGRECAN AND ITS FRAGMENTS RELEASED INTO SUPERNATANTS OF CARTILAGE EXPLANTS

To investigate the effect of further proteolytic degradation on the reactivity in the immunoassays, we treated the conditioned medium, originating from the cartilage explant cultures described above with MMP-2 (data shown) and MMP-9 (data not shown). Upon MMP-2 treatment of supernatants stimulated for 5 days with OSM/TNFα, the level of G1/G2 was reduced from 193 μg/ml to 149 μg/ml (statistically significant reduction of 23%) [Fig. 6(A)], whereas no

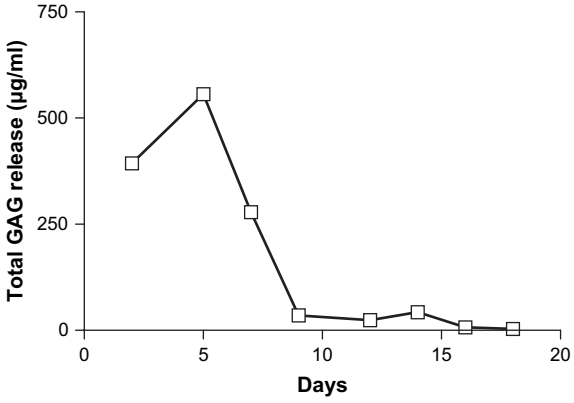

Fig. 4. The level of total GAGs (-□-) in explant cultures stimulated with OSM/TNFα.

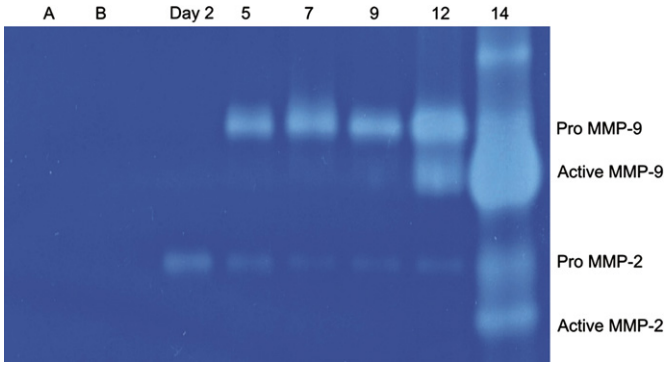

Fig. 5. Gelatinase activity investigated by zymography of conditioned medium from cartilage explants treated with OSM/TNFα. Lane A: Vehicle, conditioned medium without cytokines from day 12. Lane B: Conditioned medium from MI explants from day 12. The rest of the lanes represent conditioned medium of explants stimulated with OSM/TNFα at days 2, 5, 7, 9, 12 and 14. Active and pro-enzymes were identified by comparison to the molecular markers and MMP-2 and MMP-9 standards (not shown). The molecular weight marker showed the bands corresponding to the weights of pro MMP-9 92 kDa, active 86 kDa and pro MMP-2 72 kDa and active 66 kDa.

change was observed in the 342-G2 test [Fig. 6(C)]. When treating OSM/TNFα stimulated supernatants from day 18 with MMP-2, no difference was observed in the level of G1/G2 [Fig. 6(B)] or 342-G2 [Fig. 6(D)]. Similar data were observed by treatment of supernatants with MMP-9.

DEGRADATION OF AGGRECAN EVALUATED BY HISTOLOGY

To confirm the degradation of the matrix in the explant cultures, histology was performed on explants from day 5 and day 18 stimulated with OSM/TNFα, and proteoglycans were stained with toluidine blue. There was an intense staining of proteoglycans in non-stimulated or MI explants. However, the intensity of the staining was strongly decreased in explants stimulated with OSM/TNFα at both day 5 and day 18 [Fig. 7(A and B)].

CIRCULATING LEVELS OF AGGRECAN AND ITS FRAGMENTS IN PATIENTS WITH RA

In order to determine, if the G1/G2 and 342-G2 assays could have potential as clinical diagnostic tools, the aggrecan level was determined in healthy controls as well as individuals diagnosed with RA. The concentration of G1/G2 was significantly decreased in patients with RA compared to controls ($P = 0.0001$) [Fig. 8(A)], while the level of 342-G2 was elevated in RA individuals, though not significant [Fig. 8(B)]. To further evaluate, if there was a significant

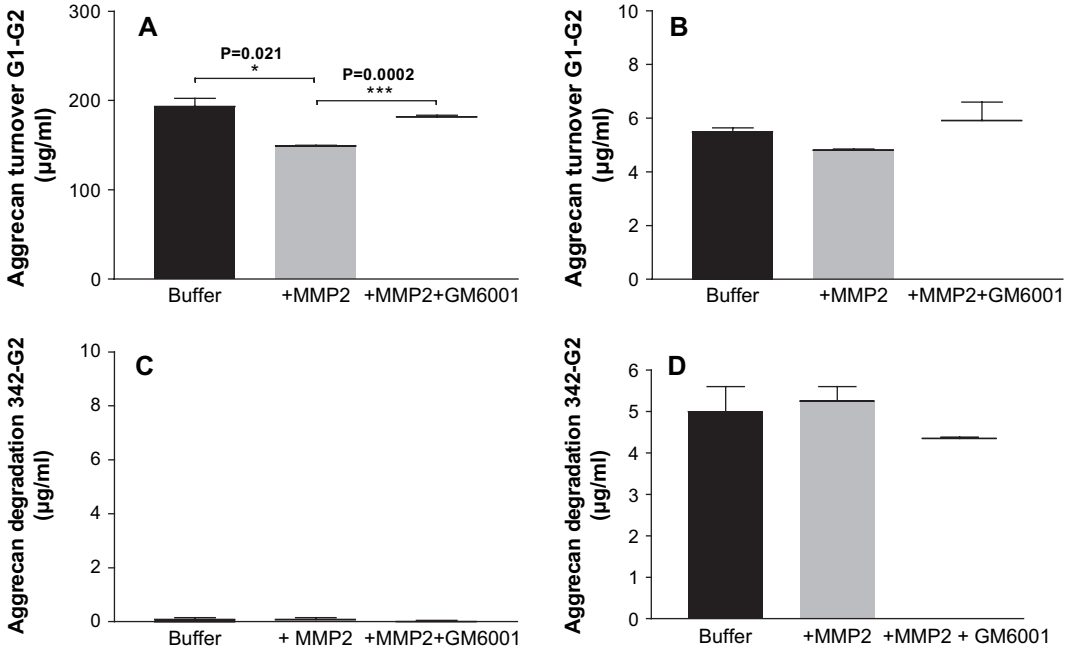

Fig. 6. Articular cartilage explants were stimulated with OSM/TNFα for 5 days (see Fig. 3) and supernatants were subsequently treated with either buffer, MMP-2 or MMP-2 with MMP inhibitor (GM6001) and measured in the G1/G2 assay (A) or 342-G2 (C). Similar supernatants from day 18 were measured in the G1/G2 (B) and 342-G2 assays (D). The values are mean + S.E.M.s, and the asterisks indicate significant differences. For the statistical analysis, unpaired *t* test with Welch's correction was used with one-tailed *P* values.

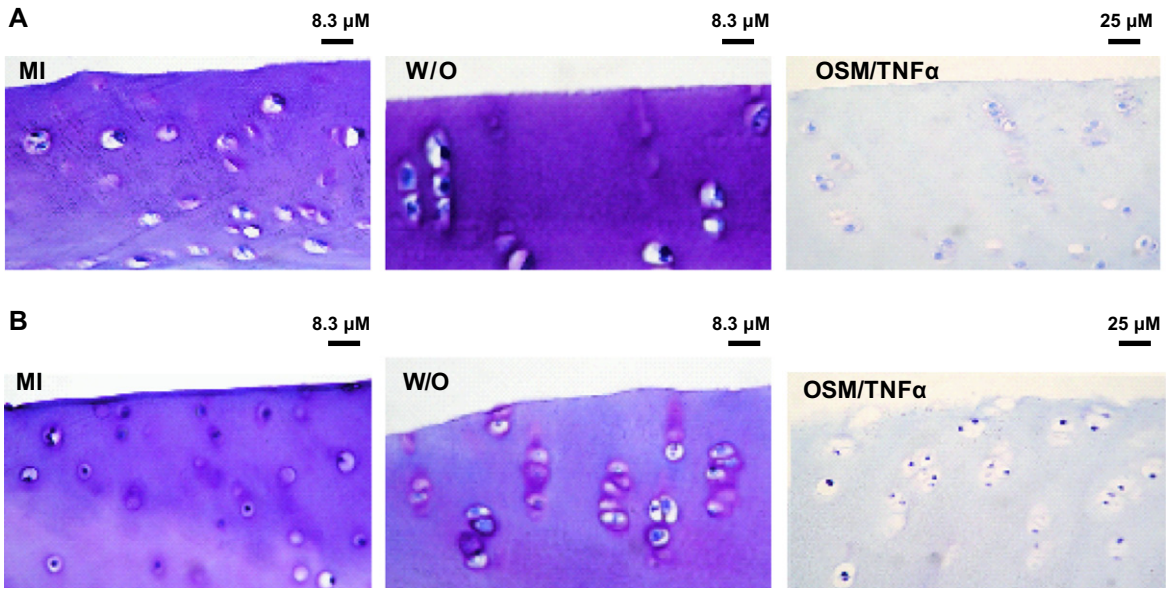

Fig. 7. Bovine articular explants were cultured in the presence (OSM/TNFα) or absence of cytokines (W/O) for 5 days (A) or 18 days (B) and subsequently fixed in formaldehyde and paraffin embedded. Sections were stained for proteoglycans by toluidine blue, and as a negative control, MI explants were also included.

difference in controls compared to RA patients in the level of degraded aggrecan of the total pool of aggrecan molecules, the aggrecan index 342-G2/G1/G2 was calculated. A significant increase ($P = 0.0074$) [Fig. 8(C)] was detected in RA individuals compared to healthy controls.

Discussion

In the present study, we have demonstrated that catabolic stimulation of bovine explant cultures leads to both MMP and non-MMP-mediated release of aggrecan fragments into the supernatant, and that these fragments can be quantitatively assessed by novel immunoassays.

To facilitate the investigation of aggrecan degradation in the cartilage matrix, new monoclonal antibodies were developed to intact aggrecan, and a series of novel immunoassays were developed. In particular, one monoclonal antibody, F-78, specific to aggrecan [Fig. 1(A)], recognizing a repetitive epitope exposed at least once on G1 [Fig. 1(B)] was selected for development of two complementary immunoassays. The G1/G2 assay employing F-78 both as a capturing and a detecting antibody quantifies intact aggrecan, as well as fragments containing G1 and/or G2, whether these fragments are being generated by MMPs or other proteases [Fig. 2(A)]. In contrast, the 342-G2 assay, using a monoclonal antibody recognizing an MMP-generated neo-epitope as a capturing antibody, detects the subpopulation of G2-containing molecules, carrying the neo-epitope ^{342}FFGVG as well [Fig. 2(B)]. Previously Fosang *et al.* used this neo-epitope specific antibody, and developed a competitive assay with a sensitivity of 7 pmol/ml[31]. The sensitivity of the 342-G2 assay is 10 ng/ml (Table I), which corresponds to 0.33 pmol/ml, and this assay is therefore around 20 times more sensitive than the competition assay. Moreover, detection of the ^{342}FFGVG neo-epitope in serum has never been reported before, implying that a highly sensitive system is required to detect this analyte.

Various *in vitro*[32–34] and *ex vivo*[14–16] models have been used for the study of cartilage metabolism. However, the articular cartilage explant model is particularly useful, as it allows the preservation of the chondrogenic phenotype. Furthermore, it is widely used as a degradation model for the study of cytokine-stimulated chondrocytes, embedded in their natural cartilage matrix, and has served as a model for the investigation of medical intervention of chondroprotective drugs[8]. In particular, the bovine explant system, stimulated with the catabolic cytokines OSM/TNFα has been used to study the pathological destruction of cartilage tissue[12,27].

In the present study, OSM/TNFα induced MMP expression was detected in the late phase, i.e., after day 12 as verified by zymography, and was observed concomitantly with the emergence of ^{342}FFGVG containing G2 aggrecan fragments in the supernatant, peaking at a concentration of around 4.5 µg/ml/mg cartilage (Fig. 3). In contrast, the initial high levels (250–350 µg/ml/mg cartilage) of G1/G2 containing analytes were reduced to background levels before MMP-2 and MMP-9 activity could be detected (Figs. 3 and 5). This suggests that the release of aggrecan molecules from the explant cultures, which can be detected in the supernatant in the G1/G2 assay, is caused by metabolic events not involving MMPs. The absence of any major effect of MMP inhibitors on the G1/G2 profile in the culture supernatants further supports this (Fig. 3). These results are consistent with other published data proving a role for ADAMTs enzymes in the early release of aggrecan from cartilage explants[15–17].

It is our hypothesis that in the supernatants at day 5, two populations of molecules exist. First, small aggrecan fragments generated by proteolytic cleavage by ADAMTs, and which contain either G1 or G2. Second, larger molecules containing both G1 and G2 are likely to be present, as further MMP cleavage reduces the read out in the G1/G2 ELISA [Fig. 6(A)]. We have used 100 nM of MMPs to investigate the further processing of analytes, while many other investigators priorly have used in the range of 0.35–2.45 nM [33,35,36]. The high concentration of MMPs will separate all FFGVG epitopes from the G2 domain, and therefore will remain undetected by the 342-G2 ELISA

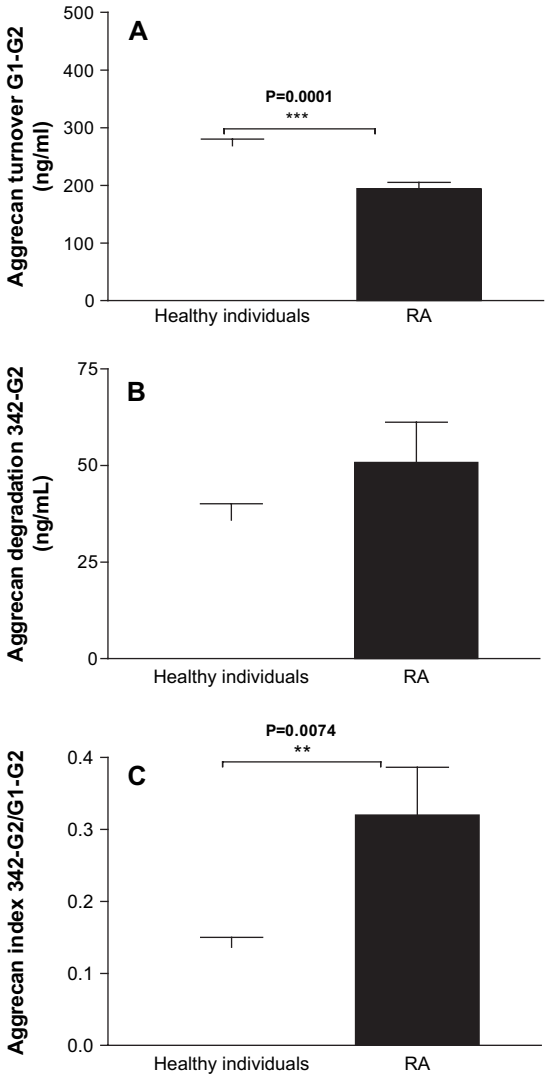

Fig. 8. Serum samples from healthy individuals ($n = 57$) and patients with RA ($n = 20$) were analyzed in the G1/G2 assay (A) and the 342-G2 assay (B). The aggrecan degradation index (C) was obtained by dividing the concentration of 342-G2 with the level measured in the G1/G2 assay for both healthy individuals and RA patients. For standardization of samples in the G1/G2 assay, intact, purified bovine aggrecan at concentrations 0.0625–1 ng/ml was used, whereas MMP treated purified porcine aggrecan at concentrations 46–3000 ng/ml was used in the 342-G2 assay. The values are mean + S.E.M.S, and the asterisks indicate significant differences. For the statistical analysis, unpaired *t* test with Welch's correction was used with one-tailed *P* values.

[Fig. 6(C)]. However, the presence of the signal at day 18 in the 342-G2 ELISA, when exogenously treating explant supernatants with MMPs [Fig. 6(D)], is explained by the lack of activity of enzymes against other minor MMP regions in IGD, once a clipping has already occurred at the IPEN^{341}–^{342}FFGV site. Mercuri et al. observed that MMP derived G2 fragments of aggrecan are indeed resistant to further aggrecanase cleavage in IGD[37], which may be the case for the MMP sites as well. Moreover, the substrate:enzyme ratio most likely has an influence on the cleavage of the IGD at the major IPEN^{341}–^{342}FFGV MMP site or multiple sites, with a high ratio in the early, and low ratio in the late phase.

When intact G1/G2 is cleaved in IGD, this will theoretically cause a 33% reduction in the G1/G2 test read out. This is because, molecules containing both G1 and G2 could bind one biotinylated antibody and 3 peroxidase labeled antibodies (due to the same repetitive epitope in G1 and G2), whereas separation of G1 and G2 produces molecules binding one peroxidase labeled antibody per 1 biotinylated antibody. Consequently, the reduction of approximately 23% in the G1/G2 test, which is observed in MMP treated supernatants could correspond to cleavage of intact G1/G2 [Fig. 6(A)].

Degradation of aggrecan has been extensively investigated through *in vivo*[15,31,38], *in vitro*[7,32–34,39], and in *ex vivo* cultures of cartilage explants[14–16,40,41]. These studies demonstrate, that besides the MMPs, a second important class of enzymes, i.e., the aggrecanases are capable of degrading aggrecan[42]. A primary site for these proteases is the sequence TEGE^{373}–^{374}ARGS in the IGD sequence[18,38,42]. Two aggrecanases, aggrecanase 1 and aggrecanase 2, also designated ADAMTS-4[15] and ADAMTS-5[15] respectively are considered to be active in the early stages of cartilage erosion in OA as well as RA[38,42]. The detection of the epitopes TEGE^{373} and ^{374}ARGS by western blotting has been reported in the early phase of explants treated with pro-inflammatory agents like TNFα, interleukin 1 or retinoic acid[12,16,40,43]. Malfait *et al.* state that when they use the MMP inhibitor XS309, the activity of ADAMTS-4 and ADAMTS-5 is not blocked at concentrations around 10 μM, but is suppressed by the ADAMTs inhibitor BB-16[18].

These studies support our observation, that the release of aggrecan fragments in the early phase is not mediated by MMPs, but rather by aggrecanases. Cleavage at the 373–374 site would generate fragments that upon treatment with MMPs cannot produce molecules recognized in the 342-G2 assay as described in the present study.

In contrast, the release of aggrecan fragments in the late phase of the OSM/TNFα stimulated explant cultures seems to be driven by MMPs, which was shown to be elevated by zymography from day 12 (Fig. 5). A steep elevation in the release of 342-G2 containing fragments was observed, and this could be completely abrogated upon incubation with an MMP inhibitor. In addition, MMP processing of the aggrecan fragments seemed to be complete in the late phase, as the addition of MMP-2 or MMP-9 to culture supernatant did not generate further 342-G2 detection [Fig. 6(D)].

The profile of the total GAG release of OSM/TNFα treated explants was similar to the trend observed in the G1/G2 assay, reaching maximum levels at day 5 (Fig. 4). However, much higher concentrations were detected in the GAG assay. This could be explained by the fact that around 90% of the molecular weight of aggrecan is made up by GAGs[2], while the rest is made up by the core protein. Other proteoglycans are detected by the GAG assay, but the contribution of these is negligible compared to aggrecan.

In order to further evaluate the G1/G2 and 342-G2 assays, our next approach was to investigate their clinical relevance. A cohort of 20 women with RA were compared to 57 healthy age-matched women. We measured serum samples in the G1/G2 and 342-G2 assays and observed a significant decrease ($P = 0.0001$) in the level of G1/G2 in RA patients compared to healthy controls [Fig. 8(A)], suggesting a suppression of total release of G1 and G2 containing aggrecan fragments. Furthermore, we showed that we could partly decrease the signal of G1/G2 after exogenously adding MMPs to explant supernatants [Fig. 6(A)]. These results support each other, as it is expected that

arthritic patients have up-regulated expression of MMPs[44,45]. This suppression of overall aggrecan turnover could be caused by the anti-inflammatory treatment in the RA patients. On the other hand, the calculated aggrecan degradation index, which is a measure of the amount of MMP degraded aggrecan compared to the total pool of aggrecan molecules (342-G2/G1/G2) showed a significant increase ($P = 0.0074$) in RA patients compared to controls [Fig. 8(C)]. The observation done by Struglics and coworkers of free G1 and G2 domains of aggrecan, as well as G1/G2 and ^{342}FFGVG-G2 molecules in different fractions of synovial fluid from OA patients, furthermore supports the existence of these molecules in the circulation of arthritic individuals[32].

Hence, in this study, we have developed the MMP-mediated and non-MMP-mediated assays 342-G2 and G1/G2. These assays hold noteworthy potential as important diagnostic tools for assessing cartilage turnover in patients diagnosed with RA, as well as the effects of intervention with potential chondroprotective agents.

Acknowledgment

We would like to thank Dr Marian Young for supplying us with the proteoglycans biglycan and decorin used for specificity investigations of the developed F-78 antibody. Furthermore, we would like to acknowledge Professor Pierre Miossec for collecting the human blood samples.

References

1. Rizkalla G, Reiner A, Bogoch E, Poole AR. Studies of the articular cartilage proteoglycan aggrecan in health and osteoarthritis. Evidence for molecular heterogeneity and extensive molecular changes in disease. J Clin Invest 1992;90:2268–77.
2. Kiani C, Chen L, Wu YJ, Yee AJ, Yang BB. Structure and function of aggrecan. Cell Res 2002;12:19–32.
3. Flannery CR, Little CB, Caterson B, Hughes CE. Effects of culture conditions and exposure to catabolic stimulators (IL-1 and retinoic acid) on the expression of matrix metalloproteinases (MMPs) and disintegrin metalloproteinases (ADAMs) by articular cartilage chondrocytes. Matrix Biol 1999;18(3):225–37.
4. Vankemmelbeke MN, Holen I, Wilson AG, Ilic MZ, Handley CJ, Kelner GS, *et al*. Expression and activity of ADAMTS-5 in synovium. Eur J Biochem 2001; 268(5):1259–68.
5. Fraser A, Fearon U, Billinghurst RC, Ionescu M, Reece R, Barwick T, *et al*. Turnover of type II collagen and aggrecan in cartilage matrix at the onset of inflammatory arthritis in humans: relationship to mediators of systemic and local inflammation. Arthritis Rheum 2003;48:3085–95.
6. Garnero P, Gineyts E, Christgau S, Finck B, Delmas PD. Association of baseline levels of urinary glucosyl-galactosyl-pyridinoline and type II collagen C-telopeptide with progression of joint destruction in patients with early rheumatoid arthritis. Arthritis Rheum 2002;46(1):21–30.
7. Little CB, Flannery CR, Hughes CE, Mort JS, Roughley PJ, Dent C, *et al*. Aggrecanase versus matrix metalloproteinases in the catabolism of the interglobular domain of aggrecan *in vitro*. Biochem J 1999;344(Pt 1):61–8.
8. Schaller S, Henriksen K, Hoegh-Andersen P, Sondergaard BC, Sumer EU, Tanko LB, *et al*. *In vitro*, *ex vivo*, and *in vivo* methodological approaches for studying therapeutic targets of osteoporosis and degenerative joint diseases: how biomarkers can assist? Assay Drug Dev Technol 2005;3:553–80.
9. Sondergaard BS, Wulf H, Henriksen K, Ostergaard K, Qvist P, Christiansen C, *et al*. Calcitonin directly attenuates collagen type II degradation by inhibition of MMP expression and activity in articular chondrocytes. Osteoarthritis Cartilage 2005;13(Suppl A):S12.
10. Billinghurst RC, Wu W, Ionescu M, Reiner A, Dahlberg L, Chen J, *et al*. Comparison of the degradation of type II collagen and proteoglycan in nasal and articular cartilages induced by interleukin-1 and the selective inhibition of type II collagen cleavage by collagenase. Arthritis Rheum 2000;43:664–72.
11. Christgau S, Garnero P, Fledelius C, Moniz C, Ensig M, Gineyts E, *et al*. Collagen type II C-telopeptide fragments as an index of cartilage degradation. Bone 2001;29(3):209–15.
12. Morgan TG, Rowan AD, Dickinson SC, Jones D, Hollander AP, Deehan D, *et al*. Human nasal cartilage responds to oncostatin M in combination with interleukin 1 or tumour necrosis factor alpha by the release of collagen fragments via collagenases. Ann Rheum Dis 2006;65:184–90.
13. van de Lest CH, van den Hoogen BM, van Weeren PR. Loading-induced changes in synovial fluid affect cartilage metabolism. Biorheology 2000;37(1-2): 45–55.
14. Fosang AJ, Last K, Stanton H, Weeks DB, Campbell IK, Hardingham TE, *et al*. Generation and novel distribution of matrix metalloproteinase-derived aggrecan fragments in porcine cartilage explants. J Biol Chem 2000;275:33027–37.
15. Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms involved in cartilage proteoglycan catabolism. Matrix Biol 2000;19:333–44.
16. Sztrolovics R, White RJ, Roughley PJ, Mort JS. The mechanism of aggrecan release from cartilage differs with tissue origin and the agent used to stimulate catabolism. Biochem J 2002;362:465–72.
17. Chockalingam PS, Zeng W, Morris EA, Flannery CR. Release of hyaluronan and hyaladherins (aggrecan G1 domain and link proteins) from articular cartilage exposed to ADAMTS-4 (aggrecanase 1) or ADAMTS-5 (aggrecanase 2). Arthritis Rheum 2004; 50(9):2839–48.
18. Malfait AM, Liu RQ, Ijiri K, Komiya S, Tortorella MD. Inhibition of ADAM-TS4 and ADAM-TS5 prevents aggrecan degradation in osteoarthritic cartilage. J Biol Chem 2002;277:22201–8.
19. Little CB, Hughes CE, Curtis CL, Janusz MJ, Bohne R, Wang-Weigand S, *et al*. Matrix metalloproteinases are involved in C-terminal and interglobular domain processing of cartilage aggrecan in late stage cartilage degradation. Matrix Biol 2002;21:271–88.
20. Fosang AJ, Last K, Gardiner P, Jackson DC, Brown L. Development of a cleavage-site-specific monoclonal antibody for detecting metalloproteinase-derived aggrecan fragments: detection of fragments in human synovial fluids. Biochem J 1995;310(Pt 1): 337–43.
21. Pratta MA, Su JL, Leesnitzer MA, Struglics A, Larsson S, Lohmander LS, *et al*. Development and characterization of a highly specific and sensitive sandwich ELISA

for detection of aggrecanase-generated aggrecan fragments. Osteoarthritis Cartilage 2006;1–11.
22. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31: 315–24.
23. Prevoo ML, van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
24. van der Heide A, Remme CA, Hofman DM, Jacobs JW, Bijlsma JW. Prediction of progression of radiologic damage in newly diagnosed rheumatoid arthritis. Arthritis Rheum 1995;38(10):1466–74.
25. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. 1975. J Immunol 2005;174:2453–5.
26. Fosang AJ, Hardingham TE. Isolation of the N-terminal globular protein domains from cartilage proteoglycans. Identification of G2 domain and its lack of interaction with hyaluronate and link protein. Biochem J 1989; 261:801–9.
27. Bonnet F, Dunham DG, Hardingham TE. Structure and interactions of cartilage proteoglycan binding region and link protein. Biochem J 1985;228:77–85.
28. Hui W, Rowan AD, Richards CD, Cawston TE. Oncostatin M in combination with tumor necrosis factor alpha induces cartilage damage and matrix metalloproteinase expression *in vitro* and *in vivo*. Arthritis Rheum 2003;48:3404–18.
29. Bai S, Thummel R, Godwin AR, Nagase H, Itoh Y, Li L, *et al*. Matrix metalloproteinase expression and function during fin regeneration in zebrafish: analysis of MT1-MMP, MMP2 and TIMP2. Matrix Biol 2005;24(4): 247–60.
30. Wu WT, Chen CN, Lin CI, Chen JH, Lee H. Lysophospholipids enhance matrix metalloproteinase-2 expression in human endothelial cells. Endocrinology 2005 Aug;146(8):3387–400.
31. Fosang AJ, Last K, Maciewicz RA. Aggrecan is degraded by matrix metalloproteinases in human arthritis. Evidence that matrix metalloproteinase and aggrecanase activities can be independent. J Clin Invest 1996;98:2292–9.
32. Fosang AJ, Neame PJ, Hardingham TE, Murphy G, Hamilton JA. Cleavage of cartilage proteoglycan between G1 and G2 domains by stromelysins. J Biol Chem 1991 Aug 25;266(24):15579–82.
33. Mercuri FA, Doege KJ, Arner EC, Pratta MA, Last K, Fosang AJ. Recombinant human aggrecan G1–G2 exhibits native binding properties and substrate specificity for matrix metalloproteinases and aggrecanase. J Biol Chem 1999;274:32387–95.
34. Tortorella MD, Liu RQ, Burn T, Newton RC, Arner E. Characterization of human aggrecanase 2 (ADAM-TS5): substrate specificity studies and comparison with aggrecanase 1 (ADAM-TS4). Matrix Biol 2002; 21:499–511.
35. Fosang AJ, Last K, Knäuper V, Murphy G, Neame PJ. Degradation of cartilage aggrecan by collagenase-3 (MMP-13). FEBS Lett 1996;380:17–20.
36. Struglics A, Larsson S, Pratta MA, Kumar S, Lark MW, Lohmander LS. Human osteoarthritis synovial fluid and joint cartilage contain both aggrecanase- and matrix metalloproteinase-generated aggrecan fragments. Osteoarthritis Cartilage 2006 Feb;14(2):101–13.
37. Mercuri FA, Maciewicz RA, Tart J, Last K, Fosang AJ. Mutations in the interglobular domain of aggrecan alter matrix metalloproteinase and aggrecanase cleavage patterns. Evidence that matrix metalloproteinase cleavage interferes with aggrecanase activity. J Biol Chem 2000;275:33038–45.
38. Janusz MJ, Little CB, King LE, Hookfin EB, Brown KK, Heitmeyer SA, *et al*. Detection of aggrecanase- and MMP-generated catabolic neoepitopes in the rat iodoacetate model of cartilage degeneration. Osteoarthritis Cartilage 2004;12:720–8.
39. Fosang AJ, Last K, Fujii Y, Seiki M, Okada Y. Membrane-type 1 MMP (MMP-14) cleaves at three sites in the aggrecan interglobular domain. FEBS Lett 1998;430:186–90.
40. Sandy JD, Neame PJ, Boynton RE, Flannery CR. Catabolism of aggrecan in cartilage explants. Identification of a major cleavage site within the interglobular domain. J Biol Chem 1991;266(14):8683–5.
41. Sztrolovics R, Alini M, Roughley PJ, Mort JS. Aggrecan degradation in human intervertebral disc and articular cartilage. Biochem J 1997;326(Pt 1):235–41.
42. Nagase H, Kashiwagi M. Aggrecanases and cartilage matrix degradation. Arthritis Res Ther 2003;5(2): 94–103.
43. Caterson B, Flannery CR, Hughes CE, Little CB. Mechanisms of proteoglycan metabolism that lead to cartilage destruction in the pathogenesis of arthritis. Drugs Today (Barc) 1999;35(4-5):397–402.
44. Brennan FM, Browne KA, Green PA, Jaspar JM, Maini RN, Feldmann M. Reduction of serum matrix metalloproteinase 1 and matrix metalloproteinase 3 in rheumatoid arthritis patients following anti-tumour necrosis factor-alpha (cA2) therapy. Br J Rheumatol 1997;36:643–50.
45. Green MJ, Gough AK, Devlin J, Smith J, Astin P, Taylor D, *et al*. Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis. Rheumatology (Oxford) 2003;42:83–8.