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**Human osteoarthritis synovial fluid and joint cartilage contain both
aggrecanase and matrix metalloproteinase generated aggrecan fragments**

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Summary

Objective: To identify the major aggrecanase and matrix metalloproteinase (MMP) generated aggrecan fragments in human osteoarthritis (OA) synovial fluid and in human OA joint cartilage.

Method: Aggrecan fragments were prepared by CsCl gradient centrifugation. Fragment distributions were compared with aggrecanase-1 (ADAMTS-4) and MMP-3 digested human aggrecan by analysis with neoepitope antibodies and an anti-G1 domain antibody, using Western immuno-blots.

Results: The overall fragment pattern of OA synovial fluid aggrecan was similar to the fragment pattern of cartilage aggrecan cleaved *in vitro* by ADAMTS-4. However, multiple glycosaminoglycan (GAG) containing aggrecanase and MMP generated aggrecan fragments were identified in OA synovial fluid and some of these fragments were produced by action of both types of proteinases. The synovial fluid content of large size aggrecan fragments with ³⁷⁴ARGS- and ³⁴²FFGV- N-terminals was about 107 and 40 pmoles per ml, respectively, out of a total concentration of aggrecan fragments of about 185 pmoles per ml. OA synovial fluid contained insignificant amounts of the G1-IPEN³⁴¹ fragment as compared to the G1-TEGE³⁷³ fragment, while OA cartilage contained significant amounts of both fragments. OA cartilage contained several GAG-containing aggrecan fragments with N-terminals of G1- or ³⁴²FFGV- but no fragments with an N-terminal of ³⁷⁴ARGS-.

Conclusions: The overall pattern of aggrecan fragments in human OA synovial fluid and cartilage supports an important role for aggrecanase in aggrecan degradation. However, the fragment patterns and their differential distribution between cartilage and synovial fluid are consistent with the existence of at least two proteolytic pathways for aggrecan degradation in human OA, generating both ³⁴²FFGV- and ³⁷⁴ARGS- fragments.

Key words: *Osteoarthritis, cartilage, synovial fluid, aggrecan, aggrecanase, matrix metalloproteinase*

Running head: *Aggrecan fragments in human OA*

Introduction

The gradual destruction of joint cartilage is a prominent feature of OA, involving loss from the tissue of the major matrix components type II collagen and aggrecan. Aggrecan depletion in arthritic joints is largely due to fragmentation of the core protein by proteolysis. The involvement of aggrecanase in this process in human arthritis was first shown by the identification in human arthritic synovial fluids of high molecular weight aggrecan fragments bearing the N-terminal sequence ³⁷⁴ARGS-^{1,2} ^α.

Multiple matrix metalloproteinase and aggrecanase cleavage sites in the articular cartilage aggrecan have since been identified by *in vivo* and *in vitro* studies of animal and human samples (Fig. 1). Three MMP cleavage sites are present in the interglobular domain (IGD), Asn³⁴¹-Phe³⁴² – being the predominant MMP cleavage site⁴⁻⁷. One MMP cleavage site has been identified between the G2 domain and the keratan sulfate enriched region (KS)⁸, together with several sites in the chondroitin sulfate enriched region one (CS1)⁹. Several aggrecanase cleavage sites have been identified in the aggrecan core protein: one in the IGD¹⁰⁻¹² at Glu³⁷³-Ala³⁷⁴, other sites are located in the chondroitin sulfate enriched region two (CS2)^{10, 11}.

Only a limited number of studies have used human synovial fluid^{1, 2, 6, 13, 14} and human joint cartilage^{4, 6, 14, 15} to characterize the aggrecan fragments generated in OA. While much of the current data suggest that destructive aggrecanolysis (i.e. in IGD) is due to aggrecanase activity^{1, 2, 6, 14}, with ADAMTS-5 playing a lead role in mouse cartilage^{16, 17}, other studies suggest that variable but quantitatively not well determined proportions of the IGD cleavage may be due to activity of MMP or other proteases^{13, 15}. Further, deletion of the ADAMTS-5 catalytic domain in

^αThe superscript numbers denote the amino acid residue positions. Human aggrecan residue numbers used in this paper were obtained by subtraction of 19 amino acids (leader sequence) from the total (1-2415 amino acid) sequence of human aggrecan (NCBI accession nr P16112³).

genetically modified mice provided a partial but not complete protection against cartilage destruction and aggrecan loss in OA and arthritis models^{16,17}. A better understanding of the relative roles of the different proteolytic pathways is highly relevant for current efforts to develop disease-modifying treatments for human OA.

In the present study, we have identified aggrecan fragments in pooled human OA cartilage and in pooled OA human synovial fluid from a large number of patients with varying stages of OA. A well-characterized set of antibodies was used to detect and quantify both aggrecanase- and MMP-generated aggrecan fragments. We detect for the first time in human samples large glycosaminoglycan containing fragments with an MMP-generated N-terminus of ³⁴²FFGV- both in OA synovial fluid and OA cartilage, and propose mechanisms for aggrecan degradation in human knee OA that incorporate the identified degradation patterns.

Materials and Methods

MATERIALS

Alcian Blue 8GS (C.I. no 742240) was from Chroma-Gesellschaft (Köngen, Germany). 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 6-aminohexonic acid (EACA), Benzamidine-HCl, BSA, Chondroitin sulfate type C from shark cartilage (C4384), Na₄-EDTA, N-ethylmaleimide (NEM), trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), Iodoacetamide, o-phenathroline, Pepstatin A and Phenylmethylsulfonyl fluoride (PMSF) were from Sigma. Chondroitinase ABC (EC 4.2.2.4), keratanase (EC 3.2.1.103) and keratanase II (from *Bacillus sp. Ks36*) were from Seikagaku. Molecular weight markers 10-250 kDa (Precision Plus Protein Standards) were from BioRad, and for a 400 kDa molecular weight marker reduced laminin from mouse Engelbreth-Holm Sarcoma (Roche) was used.

Activated recombinant human MMP-3 was provided by Merck. Human recombinant ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs, aggrecanase-1) was provided by GlaxoSmithKline¹⁸. ECL Plus detection and Hyperfilm-ECL were from Amersham Biosciences. Mini gels (4-12 % and 3-8 %), LDS sample buffer, SDS running buffers (Tris-Acetate and MOPS buffers), transfer buffer were all NuPAGE[®] from Invitrogen. PVDF membranes were from Invitrogen. Non-fat dry milk (Semper) was from the local supermarket. Neo-specific rabbit anti-peptide sera anti-KEEE, anti-FFGV, anti-LGQR, anti-TEGE and anti-IPEN were prepared at Merck. Neo-specific anti-ARGS monoclonal antibody (mab OA-1) was raised in mouse against the synthetic peptide ARGSVILTVK (GlaxoSmithKline). Neo-specific polyclonal anti-SELE antibody was raised in rabbit against the synthetic peptide CASTASELE (GlaxoSmithKline). Anti-G1 antibody (polyclonal rabbit anti ATEGQV- peptide sera) was a gift from Dr. John Sandy (Shriners Hospital and University of South Florida, Tampa). Polyclonal anti-aggrecan-G3 antibody was from Affinity BioReagents (Golden, CO., USA). Anti-chondroitin sulfate (CS) antibody (mab 3-B-3) was from Seikagaku. Peroxidase conjugated secondary antibodies goat anti-mouse IgG was from Cell Signaling Technology, goat anti-rabbit IgG was from KPL and goat anti-mouse IgM was from Sigma. Synthetic peptides used in immuno-blocking experiments: neoepitope peptide ARGSVILTVKGGC, neoepitope peptide CASTASELE and spanning peptide DIPENFFGVGGEEEDC were from GlaxoSmithKline; spanning peptide EVVTASTASELEGRGT, neoepitope peptide FFGVGGEEEDITVC, neoepitope peptide CEVAPTTFKEEE and spanning peptide VAPTTFKEEEGLGS were from Innovagen (Lund, Sweden); spanning peptide EGARGSVILTVKPIF was from Merck; anti-G1 blocking peptide CATEGQVRVNSIYQDKVSL was a kind gift from Dr. John Sandy.

HUMAN CARTILAGE AND SYNOVIAL FLUID SAMPLES

Knee cartilage was obtained from patients undergoing joint replacement surgery for OA. All remaining cartilage was removed from 10 knee joints, diced and pooled for aggrecan extraction. Samples were stored at -80°C . Synovial fluid samples from more than 100 patients with advanced knee OA or with varying stages of post-injury cartilage changes were pooled and then stored at -80°C . The synovial fluids and the cartilage samples were not from the same subjects. All procedures were approved by the ethics review committee of the Medical Faculty of Lund University.

AGGREGAN ISOLATION FROM SYNOVIAL FLUID

Human synovial fluid pool (in 50 mM Na-acetate, pH 6.8) was cleared by centrifugation (20 400xg, 35 min, 4°C), and guanidine-HCl (4 M final concentration) and CsCl (starting density 1.5 g/ml) was added to the supernatant. A CsCl density gradient centrifugation was performed (162 000xg, 48 h, 16°C) and the D1-fraction (bottom two-fifth) was collected, dialyzed against Millipore-water (Type I, 18.2 M Ω ·cm), and freeze-dried. This sample, called SF-D1, was stored at -20°C . An associative (without guanidine-HCl) A1-fraction, called SF-A1, was also made from the human synovial fluid pool, using the same method and the same starting density. Both the associative and the dissociative preparations were conducted either in the absence or in the presence of proteinase inhibitors, 10 mM Na₄-EDTA, 0.4 mM AEBSF, 1 μM Pepstatin A, 5 μM E64 and 5 mM o-phenathroline (in gradient centrifugation) and 10 mM Na₄-EDTA, 0.4 mM PMSF, 1 μM Pepstatin A, 100 μM Iodoacetamide and 2 mM o-phenathroline (in dialysis).

AGGREGAN ISOLATION FROM CARTILAGE

Aggrecan from a knee cartilage pool of ten OA patients was extracted with guanidine-HCl (4 M) in the presence of proteinase inhibitors (10 mM Na₄-EDTA, 100 mM EACA, 10 mM NEM, 5 mM Benzamidine-HCl and 5 mM PMSF) and then isolated by associative-dissociative CsCl density gradient centrifugation, in the presence of the proteinase inhibitors, as described¹⁵. No hyaluronan (HA) was added. Fractions A1D1 and A1D3 were collected (herein called cartilage-A1D1 and cartilage-A1D3) and finally dialyzed against either Millipore-water or against Millipore water containing proteinase inhibitors (5 mM Na₄-EDTA, 10 mM EACA, 2 mM NEM, 2 mM Benzamidine-HCl and 0.4 mM PMSF) prior freeze drying.

GLYCOSAMINOGLYCAN AND PROTEIN QUANTITATION

The method for quantitation of glycosaminoglycan (GAG) by Alcian Blue precipitation was modified from Björnsson¹⁹. Samples and chondroitin sulfate standards (75 µl) were precipitated for 2 h at 4°C with 0.04 % w/v Alcian Blue, 0.72 M guanidine-HCl, 0.25 % w/v Triton X-100, 0.1 % v/v H₂SO₄ (0.45 ml). The precipitates were collected as pellets at 16 000xg 15 min, 4°C and then dissolved in 4 M guanidine-HCl, 33 % v/v 1-propanol (0.25 ml) prior to absorbance measurement.

The protein quantitation method was modified from the Pierce Micro BCA™ Assay – microplate procedure. Samples and bovine serum albumin standards were incubated with bicinchoninic acid (BCA) on a 96-well microtiter plate at 60°C for 1 h. After room temperature equilibration the absorbance was measured at 570 nm with a plate reader.

PROTEINASE DIGESTION OF CARTILAGE AGGREGAN *IN VITRO*

Human aggrecan cartilage-A1D1 (1.5 nmol) was digested for 16 h at 37°C with recombinant human MMP-3 (0.353 nmol) in proteinase digestion buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl₂, pH 7.5). Human aggrecan cartilage-A1D1 (856 pmol) was digested for 3 h at 37°C with recombinant human ADAMTS-4 (5.13 pmol) in the same buffer. The enzymatic digestion reactions (1 ml) were stopped by addition of EDTA (18.3 μmol) and were directly deglycosylated (see below). The molar calculations were based on a dry weight molecular mass for human aggrecan assuming 1000 kDa, for MMP-3 the molecular weight (M_w) used was 42.8 kDa and for ADAMTS-4 we used an M_w of 60 kDa.

DEGLYCOSYLATION

Aggrecan in deglycosylation buffer (50 mM Tris-acetate, 50 mM Na-acetate, 10 mM EDTA pH 7.6) was first digested for 2 h at 37°C with chondroitinase ABC (1 mU/μg GAG), and then digested for another hour at 37°C with addition of keratanase (1 mU/μg GAG) in the presence of 1 mM AEBSF and 10 mM NEM. Finally, the aggrecan was digested at pH 6.0 for 1 h at 37°C with addition of keratanase II (0.1 mU/μg GAG). The deglycosylated samples were dried in a vacuum centrifuge and dissolved in 2x concentrated sample buffer.

WESTERN IMMUNO-BLOT ANALYSIS

Deglycosylated samples (0.5-5 μg GAG per 5 mm well) were denatured in 1x sample buffer under reducing conditions (50 mM DTT) by boiling for 5 min. Samples were separated (according to manufacturer's instructions) by SDS-PAGE on either 3-8 % Tris-Acetate gels or on

4-12 % Bis-Tris gels. Proteins were electrophoretically transferred at room temperature at 40 V for 70 min (from 3-8 % gels) or at 35 V for 65 min (from 4-12 % gels) onto PVDF membranes (0.2 μm). After transfer, membranes were dried completely at room temperature. Immuno-reactions were done according to the ECL Plus instruction manual (Amersham Biosciences) using film for detection. Membranes were washed in TBST-150 (20 mM Tris-HCl, 150 mM NaCl, 0.1 % v/v Tween 20, pH 7.6) four times at each step using 4 ml/cm². Blocking was conducted in blocking buffer (5 % w/v non-fat dried milk in TBST-150), and antibody incubations were done in blocking buffer for 1 h at room temperature. For the anti-G1 immuno-reaction, the washes were conducted in TBST-300 (20 mM Tris-HCl, 300 mM NaCl, 0.1 % v/v Tween 20, pH 7.6), blocking was conducted in 3 % w/v BSA (in TBST-300), and antibody incubations were conducted in 1 % w/v BSA (in TBST-300). The following primary antibodies were used: anti-KEEE (1:2000 or 1:5000 dilution), anti-FFGV (1:1000 or 1:2000 dilution), anti-LGQR (1:500 or 1:1000 dilution), anti-TEGE (1:3000 dilution), anti-IPEN (1:10 000 dilution), anti-ARGS (1:4000 dilution, 2.7 $\mu\text{g/ml}$), anti-SELE (1:1000 dilution, 1.2 $\mu\text{g/ml}$), anti-G1 (1:2000 or 1:5000 dilution, anti-G3 (1:500 dilution) and anti-CS stubs (1:3000 dilution). The following secondary peroxidase conjugated antibodies were used; goat anti-mouse IgG (1:50 000 and 1:15 000 dilutions, 2.5 or 0.75 ng/ml), goat anti-rabbit IgG (1:400 000 and 1:75 000 dilutions 2.5 or 13.3 ng/ml) and goat anti-mouse IgM (1:100 000 dilution). In the peptide blocking experiments the peptide and the antibody were incubated simultaneously with the blotted proteins.

QUANTIFICATION OF FFGV- AND ARGS-FRAGMENTS

The complete conversion of GAG containing G1-fragments to either FFGV- or ARGS-fragments (with corresponding G1-IPEN and G1-TEGE fragments) was achieved by digesting (as

described above) cartilage A1D1-fraction for 16h with MMP-3 or by digesting for 24h with ADAMTS-4. These samples were deglycosylated and used as FFGV- or ARGS-standards in the Western-blot quantification. The standards were assumed to contain one nmol FFGV- or ARGS-fragments per mg aggrecan dry weight, using a Mw of 1×10^6 g/mol aggrecan. The synovial fluid sample (D1-fraction) and standards were probed by anti-ARGS or anti-FFGV antibodies as described above. The detection and quantification was conducted in a luminescence image analyzer (Fujifilm LAS-1000) using Image Gauge version 4.0 (Fujifilm) software. Samples and standards were used within a linear range of the imaging system, and the total sum of FFGV- or ARGS- immuno-signal was used in the quantification.

IDENTIFICATION OF AGGREGAN FRAGMENTS IN SYNOVIAL FLUID AND CARTILAGE

Most of the CsCl-purified aggrecan fragments were immuno-identified at both N- and C-terminal ends. Fragments that were not immuno-verified at both ends were further characterized by molecular weight calculations, based on their migration in the PAGE-system, together with a specific calibration constant (A Struglics, S Larsson, LS Lohmander unpublished). The calibration constant (K) was generated from several aggrecan fragments identified at both N- and C-terminal ends by dividing the SDS-PAGE Mw (m) with the sum of the total amino acid Mw (a) and the total Mw of GAG stubs (g): $K = m/(a + g)$.

Results

CHARACTERIZATION OF ANTIBODIES USING *IN VITRO* DIGESTED CARTILAGE AGGREGAN AND BLOCKING PEPTIDES

Several aggrecan fragments were detected in the ADAMTS-4 and MMP-3 *in vitro* digested cartilage-A1D1 samples (Fig. 2). The N- and C-terminal sequences of these aggrecan fragments were determined in matching Western immuno-blot experiments (Table I). The anti-ARGS, anti-SELE, anti-KEEE and anti-FFGV neoepitope antibody immuno-reactions were completely blocked by the specific neoepitope peptides (Fig. 2A-D). Little or no blocking of the neoepitope immuno-reactions was observed in the presence of the corresponding spanning peptides (Fig. 2A-D). The anti-G1 immuno-reactions were completely blocked by the corresponding immunization peptide (Fig. 2E), and this antibody has been further described^{20, 21}. The anti-ARGS antibody (mab OA1) has been further characterized by Western and ELISA experiments (Pratta *et al.* unpublished), whereas characterization of anti-TEGE and anti-IPEN neoepitope antibodies was published²².

Peptide crossover experiments were conducted, testing *e.g.* anti-SELE immuno-reaction in the presence of ARGSVIL-peptide. None of these experiments showed false positive immuno-blocking (results not shown).

The HRP conjugated secondary antibodies used in this paper did not bind directly to the blotted proteins in our Western immuno-blot experiment systems (results not shown). Further, the neoepitope antibodies used did not bind to human IgG, BSA, MMP-3, ADAMTS-4, deglycosylation enzymes or molecular weight markers (results not shown). The anti-G1 antibody

showed immuno-reactivity against a 100 kDa non-aggrecan polypeptide which is marked as # in Fig. 2E (see figure legend).

These findings show that the antibodies used were specific for the neoepitopes and sequences examined.

THE EFFECT OF PROTEINASE INHIBITORS DURING THE PURIFICATION AND DIALYSIS OF AGGREGAN

It was reported that guanidine-HCl denatured MMPs could be activated during dialysis against distilled water²³. Therefore, we compared aggrecan purification from human synovial fluid and cartilage in the presence or absence of proteinase inhibitors (a) in the dialysis against distilled water for cartilage aggrecan purifications, and (b) in all steps of the purification of aggrecan from synovial fluid (see Materials and Methods). The results showed similar immuno-patterns and equal fragment intensities independent of the presence or absence of proteinase inhibitors (result not shown).

These results indicate the absence of proteolysis during preparation of aggrecan fragments from joint cartilage or synovial fluid as done here. The aggrecan fragments detected in this report are thus likely to have originated from *in vivo* aggrecanolysis.

SIMILARITIES BETWEEN AGGREGAN SPECIES DETECTED IN HUMAN OA SYNOVIAL FLUID AND AGGREGAN FRAGMENTS PREPARED FROM ADAMTS-4 *IN VITRO* DIGESTED HUMAN JOINT CARTILAGE

Western analysis of unpurified synovial fluid provided little or no information on the aggrecan fragment content (result not shown), presumably due to the low concentration of GAG-containing aggrecan fragments, together with high concentration of protein and hyaluronan in synovial fluid. We therefore purified high density D1 and A1 fractions of aggrecan fragments from pooled human synovial fluid using CsCl density gradient centrifugation. Based on Western analysis using an antibody which detects the products associated with the aggrecan core protein following chondroitinase ABC digestion (mab 3-B-3), the immunoreactivity patterns of the aggrecan fragments detected in human OA synovial fluid D1-fraction showed a high degree of similarity to the ADAMTS-4 *in vitro* cleaved human cartilage-A1D1 sample, but not with an MMP-3 digested sample (Fig. 3). The aggrecan fragments detected by anti-ARGS, anti-SELE, anti-KEEE and anti-LGQR antibodies in the OA synovial fluid D1-fraction were also observed in the ADAMTS-4 *in vitro* cleaved cartilage A1D1 sample. Not only did these samples show the same patterns with the different antibodies, but the aggrecan fragments from the two samples also showed similar molecular weights (Table I). A difference observed in these comparison experiments was that the ADAMTS-4 *in vitro* digested cartilage-A1D1 sample contained a low molecular weight (51 kDa) anti-KEEE fragment, most likely ¹⁵⁴⁶GRGT-KEEE¹⁷¹⁴, which is not seen in the synovial fluid D1-fraction (Fig. 3), although this fragment was observed in other preparations of the OA synovial fluid D1-fraction (results not shown). In contrast, the aggrecan fragment pattern of the OA synovial fluid sample differed significantly from the aggrecan fragment pattern of MMP-3 *in vitro* digested cartilage-A1D1 and from the cartilage-A1D1 sample (Table I).

These results suggest that the majority of the aggrecan fragments identified in human OA synovial fluid are generated from *in vivo* aggrecanase activity.

AGGRECAN FRAGMENTS DETECTED IN HUMAN OA SYNOVIAL FLUID ARE GENERATED FROM BOTH AGGRECANASE AND MMP ACTIVITIES

GAG-containing aggrecan fragments in the OA synovial fluid (D1-fraction) were identified in Western immuno-blot experiments by using anti-G1 and neoepitope antibodies (Fig. 4). To determine the likely N- and C-terminal sequences of the aggrecan fragments, several approaches were used. Firstly, the Western immuno-blot experiments were repeated several times with the different antibodies to give average values for the molecular weights of the aggrecan fragments. Secondly, the lanes on the PVDF-membranes were divided in half after transfer, incubated with different antibodies, and physically realigned before detection.

The neoepitope antibodies verified both aggrecanase and MMP generated N-terminal fragments in the OA synovial fluid (Fig. 4). Several G1-domain containing fragments including G1-SELE¹⁵⁴⁵ (340 kDa) and G1-KEEE¹⁷¹⁴ (354 kDa) were observed. The two ³⁴²FFGV- fragments observed, 310 kDa and 335 kDa, contained aggrecanase generated C-terminal – SELE¹⁵⁴⁵ and –KEEE¹⁷¹⁴ (Fig. 4B).

The faster migrating anti-SELE immuno-band was composed of ³⁷⁴ARGS-SELE¹⁵⁴⁵ and ³⁴²FFGV-SELE¹⁵⁴⁵ fragments having similar molecular weights (Fig. 4). The multiple ³⁷⁴ARGS-CS1⁹⁷¹⁻¹³⁵² fragments (129-159 kDa) and two G1-CS1⁹⁷¹⁻¹³⁵² fragments (200 kDa and 252 kDa) had their C-terminal sequences identified as -GVED⁹⁷¹⁻¹³⁵², from the multiple MMP-3 cleavage site GVED⁹⁷¹⁻¹³⁵²↓⁹⁷²⁻¹³⁵³ISGL (see Fig. 1). Small amounts of low Mw ³⁴²FFGV- fragments (80-95 kDa) were detected in the SF-A1 fraction, while only trace amounts of these fragments were found in the SF-D1 fraction of OA synovial fluid (results not shown). Such low Mw ³⁴²FFGV- fragments have also been detected in Q-Sepharose purified synovial fluids¹³. A weakly reacting

50 kDa anti-KEEE fragment, most like $^{1546}\text{GRGT-KEEE}^{1714}$, was detected in SF-D1 and SF-A1 samples of the OA synovial fluid pool (results not shown, Table I). This fragment has previously been detected in normal human synovial fluids¹⁴.

The $^{1920}\text{LGQR-G3}$ fragment detected in SF-D1 samples was N-terminally (Fig. 3) and C-terminally (result not shown) verified, and except for the C-terminals in $^{374}\text{ARGS-}$ and G1-CS1 $^{971-1352}$ fragments and the N-terminal of the G1-KEEE 1714 fragment, all the high molecular weight SF-D1 fragments detected were immuno-verified both N- and C-terminally (Fig. 4).

These results show that OA synovial fluid contains at least nine different GAG-containing aggrecanase and MMP generated fragments. Several of these fragments were likely produced by the action of both proteinases. In contrast to other reports^{6, 14}, we detected for the first time large GAG containing $^{342}\text{FFGV-}$ fragments in OA synovial fluid.

G1-TEGE 373 AND G1-IPEN 341 FRAGMENTS WERE BOTH DETECTED IN HUMAN OA CARTILAGE, BUT ONLY INSIGNIFICANT AMOUNTS OF G1-IPEN 341 AS COMPARED TO G1-TEGE 373 WAS OBSERVED IN HUMAN OA SYNOVIAL FLUID

In order to detect G1-TEGE 373 and G1-IPEN 341 aggrecan fragments we prepared an A1D3 fraction from the OA cartilage pool (cartilage-A1D3) and an A1-fraction from the OA synovial fluid pool (SF-A1). As control samples we used ADAMTS-4 or MMP-3 *in vitro* digested cartilage-A1D1. After *in vitro* digestion of the cartilage-A1D1, a 65 kDa G1-TEGE 373 fragment was detected in the ADAMTS-4 cleaved sample, and a 52 kDa G1-IPEN 341 fragment was observed in the MMP-3 digested sample as verified by the anti-G1, anti-TEGE and anti-IPEN immuno-reactions (Fig. 5). The cartilage-A1D3 sample showed approximately equal

immunoreactivities for the 65 kDa G1-TEGE³⁷³ and the 52 kDa G1-IPEN³⁴¹ fragments, whereas the SF-A1 sample showed very low amounts of G1-IPEN³⁴¹ as compared to the G1-TEGE³⁷³ fragment (Fig. 5). Similar results were obtained from experiments using DE52 cellulose (diaminoethylcellulose) anion exchange¹⁴ purified aggrecan samples prepared from the OA synovial fluid pool (results not shown). Further, when increasing amounts of G1-IPEN³⁴¹ fragments were added to SF-A1 samples, there was a corresponding increase in IPEN-neoepitope immuno-reactivity, suggesting that the low amount of G1-IPEN³⁴¹ fragment detected in synovial fluid was not due to inhibitory factors (results not shown). The OA cartilage fraction A1D1 and the OA synovial fluid fraction D1 did not contain any G1-TEGE³⁷³ or G1-IPEN³⁴¹ fragments (results not shown).

These results, based on the –IPEN and –TEGE neoepitope antibodies together with the G1-antibody, suggest that OA synovial fluid has a very low content of the G1-IPEN³⁴¹ fragment compared to the G1-TEGE³⁷³ fragment, but that OA cartilage contains significant amounts of both fragments.

GAG CONTAINING AGGREGAN FRAGMENTS DETECTED IN HUMAN OA CARTILAGE

To detect high molecular weight GAG containing aggrecan fragments in the pooled OA cartilage an A1D1 fraction was prepared. No fragments with the N-terminal neoepitope ARGS were detected in the cartilage-A1D1 (Fig. 6) or cartilage-A1D3 samples (results not shown). On the other hand, several high molecular weight fragments with the N-terminal neoepitope FFGV were detected by Western immuno-blot in the cartilage-A1D1 sample (Fig. 6). Two FFGV containing aggrecan polypeptides were identified as a 411 kDa ³⁴²FFGV-G3 fragment (also C-terminally verified, results not shown), and as a 340 kDa ³⁴²FFGV-KEEE¹⁷¹⁴ fragment (Fig. 6 and

Table I). A 300 kDa ³⁴²FFGV- fragment had a CS1 located C-terminal identified as -DLS¹³³⁴⁻¹⁴⁵³, from the calpain-2 site²⁴ XDLS¹³³⁴⁻¹⁴⁵³↓¹³³⁵⁻¹⁴⁵⁴GLPS (Fig. 6 and Table I). In addition, several high molecular weight G1 fragments were observed in the cartilage-A1D1 sample. Besides the 429 kDa full length G1-G3 molecule (also verified C-terminally, results not shown), the cartilage-A1D1 sample also contained a 367 kDa G1-KEEE¹⁷¹⁴ fragment, a 331 kDa G1-SELE¹⁵⁴⁵ fragment and two (197 and 217 kDa) G1-GVED⁹⁷¹⁻¹³⁵² (-CS1) fragments (Fig. 6 and Table I). The 100 kDa false-positive anti-G1 immuno-band, derived from chondroitinase ABC polypeptide (Fig. 2), was also observed in the cartilage-A1D1 sample (Fig. 6). No N-terminal LGQR fragments were observed in these cartilage samples (results not shown).

These results show that several high molecular weight GAG containing fragments with N-terminal sequences of either G1- or ³⁴²FFGV-, but not ³⁷⁴ARGS-, are present in the OA cartilage.

FFGV- AND ARGS-FRAGMENTS WERE QUANTIFIED IN HUMAN OA SYNOVIAL FLUID

OA synovial fluid (D1-fraction) was quantified for content of FFGV- or ARGS-fragments using FFGV- and ARGS-standards, generated by MMP-3 or ADAMTS-4 total aggrecan cartilage-A1D1 digests (as an example see Fig. 2A and 2D), using Western immuno-blot and a luminescence imaging system. The OA synovial fluid contained 40.3 pmol FFGV- and 107 pmol ARGS-fragments per ml neat synovial fluid, with an ARGS- to FFGV-fragment molar ratio of 2.7 (Table II). The GAG concentration in the human synovial fluid pool was 140 µg per ml. Based on the assumption that 75 % of the aggrecan Mw represents GAG, the total amount of aggrecan fragments per ml SF was 185 µg, or 185 pmoles assuming an Mw for aggrecan of 1x10⁶ g/mol. Based on this quantification, 20-25% of the GAG-containing aggrecan fragments in

pooled human OA synovial fluid carried the FFGV- N-terminal sequence, while the corresponding proportion carrying the ARGS-terminal was 55-60%.

Discussion

Our understanding of aggrecan degradation pathways *in vivo* in human OA is based on samples from a small number of patients^{1, 2, 6, 13, 14}. By pooling synovial fluids from more than a hundred OA patients, we have identified the major aggrecan fragments present in knee OA synovial fluid, independent of disease stage, age, and other variables that might influence fragment patterns in the individual patient. Due to ethical constraints the human joint cartilage used was limited to that from knee replacement surgery, and subject origin was separate from the origin of the synovial fluid pool. However, the joint cartilage pool included a range of samples from the macroscopically normal to that with advanced OA pathology. The OA synovial fluid results presented in this paper were obtained from several D1- and A1-fraction preparations, and the OA cartilage data were obtained from two separate A1D1/A1D3 preparations, all providing reproducible results. Using Western immunoblot, we detected no differences in the aggrecan fragment patterns or intensities of cartilage and synovial fluid samples when the purification was done in the presence or absence of proteinase inhibitors. This contrasts to a previous report, where aggrecanolysis by MMPs was observed during the dialysis step in the absence of proteinase inhibitors when guanidine-HCl was removed from extracts of cultured pig cartilage²³. However, the cartilage explants used in that study were stimulated *in vitro* with IL-1, which would be expected to significantly upregulate protease expression and activity.

All the antibodies used were carefully tested for false positive recognition. We characterized the aggrecan fragments by identification of both N- and C-terminal sequences using Western immuno-blot and by size calculation based on electrophoretic migration.

Although the OA synovial fluid contained several aggrecan fragments generated by both aggrecanases and MMPs, and some that were generated only by MMP (Table I), it is evident from the Western immuno-blots that the aggrecan fragment pattern in OA synovial fluid was mainly generated by aggrecanase activity (Fig. 3; Table I). It has been suggested that ADAMTS-4 at high concentrations *in vitro* may cleave aggrecan at Asn³⁴¹-Phe³⁴², in addition to the preferred Glu³⁷³-Ala³⁷⁴ site²⁵. At the protease concentrations used in the present study we were unable to replicate these findings (Table I).

The differential distribution of aggrecan fragments between OA cartilage and OA synovial fluid (Table I, Fig. 7) may be caused by several different, but not mutually exclusive, processes, including but not limited to those discussed here. *Firstly*, aggrecan structure is variable. For example, newly synthesized molecules are likely to have an intact G3 domain while older aggrecan molecules appear to be increasingly truncated at the C-terminal end. This heterogeneity of the aggrecan core protein will influence the behavior of fragments once cleaved, for example through interaction of the G3 domain with other matrix components²⁶. Further, variability of aggrecan carbohydrate substitution may affect protease susceptibility²⁷. It is unclear to what extent aggregation varies, but this may also influence aggrecan fragment behavior. *Secondly*, with more than one protease involved in degradation, there is potential for differential protease activation and substrate specificity, the latter influenced by the order of cleavage. Further potential for variability in aggrecan degradation pathways may be provided by C-terminal processing of ADAMTS-4 which was shown to influence its binding to extracellular matrix

components, and substrate specificity²⁸. ADAMTS-4 is inhibited by TIMP-3 and by interaction with fibronectin, providing yet other possibilities for protease regulation^{29,30}. *Thirdly*, the differential distribution of aggrecan subpopulations and proteolytic activities within the cartilage matrix and synovial fluid will likely play an important role in generating the observed distribution. For example, molecules of different ages may be differentially distributed within the cartilage matrix, and newly synthesized molecules with an intact G3 domain may be preferentially located close to the chondrocytes in the territorial matrix, while older, truncated molecules may dominate in the interterritorial matrix. *Fourthly*, cellular uptake mechanisms may differentiate between different molecular fragments.

Since we detected in human OA cartilage both aggrecanase-generated G1-TEGE³⁷³ and MMP-generated G1-IPEN³⁴¹ fragments (Fig. 5), their distal counterparts, carrying the neopeptides ³⁷⁴ARGS- and ³⁴²FFGV-, must both have been produced in the tissue. There was, however, a striking difference in the distribution of these fragments between cartilage and synovial fluid. The MMP-generated ³⁴²FFGV-fragments were partly retained in cartilage (Tables I, II; Figs. 6, 7), whereas the aggrecanase generated ³⁷⁴ARGS- fragments were not found in the tissue but only in the synovial fluid (Table I; Figs. 4A, 6, 7). Quantification showed that the OA synovial fluid pool contained 107 pmoles per ml of ³⁷⁴ARGS- fragments (Table II). This amount corresponds to approximately 60 % of the total amount of aggrecan fragments in human OA synovial fluid (estimated to 185 pmoles/ml), and corroborates earlier findings in arthritic synovial fluids^{1,2}.

Although the possibility exists that the ³⁷⁴ARGS-fragments detected in the synovial fluid are the result of aggrecanase activity in the joint cavity, we suggest that these fragments are a product of aggrecanase cleavage of molecules within the cartilage that lack the G3-domain.

When such G3-truncated fragments are detached from the HA interacting G1-domain by aggrecanase cleavage, diffusion into the joint cavity may occur. Consistent with this proposal, all the ³⁷⁴ARGS-fragments detected in the OA synovial fluid lacked the G3-domain (Figs. 4, 7). Cartilage explant experiments have shown that ³⁷⁴ARGS-fragments readily migrate out into the culture media, resulting in very low ³⁷⁴ARGS-neoepitope content in the tissue^{6,31}. On the other hand, a possible association between the KS enriched regions of aggrecan and type II collagen has been reported^{32,33}, which could act to retain these fragments in the cartilage. Endocytosis by chondrocytes of ³⁷⁴ARGS-neoepitope fragments may also occur, although an HA-G1 complex is required for the endocytosis³⁴.

The partial retention in the cartilage of the large ³⁴²FFGV-G3 fragments (Figs. 6, 7), where the G3 domain may interact with fibulin-2 and other matrix molecules²⁶, could be explained if aggrecan molecules attacked by MMPs were preferentially those newly synthesized and still retaining their G3 domain. It is more difficult to explain the matrix retention of the G3-truncated FFGV-fragments ³⁴²FFGV-CS1 and ³⁴²FFGV-KEEE¹⁷¹⁴ (Fig. 6). Since ³⁷⁴ARGS-fragments of similar sizes are detected in synovial fluid, but not in the cartilage (Table I; Fig. 6), neither the G2-domain nor the KS and CS enriched regions seem to mediate this tissue retention. The ³⁴²FFGV-neoepitope itself or the N- and O-linked substitutions located between Phe³⁴² and Glu³⁷³ within the IGD could be the mediators of this retention^{35,36}. In support, synovial fluid low Mw ³⁴²FFGV- fragments (80-95 kDa) migrated in the associative CsCl gradient to the bottom A1-fractions (Table I), which indicates an association between these fragments and HA or other molecules binding to HA. Partial matrix retention of G3-truncated ³⁴²FFGV- fragments has been reported³⁷. Previously detected FFGV fragments in human OA synovial fluid^{13,38} and in porcine cartilage³⁷ were a mixture of several smaller peptides ranging between 40-200 kDa. In contrast,

we detected large (> 300 kDa) 342 FFGV-aggrecan fragments in the OA synovial fluid and OA cartilage (Figs. 4B, 6). The level of the large 342 FFGV-aggrecan fragments in the OA synovial fluid pool was about 40 pmol per ml, or 20-25% of the total amount of aggrecan fragments (Table II). This compares with previous findings of 10-20 pmol per ml OA synovial fluid, where a competitive ELISA using the FFGV epitope mab AF28 was used to detect low Mw FFGV-fragments¹³.

We readily detected both G1-TEGE³⁷³ and G1-IPEN³⁴¹ fragments in OA cartilage (Fig. 5), similar to previous reports using Western analysis^{6, 14, 15} or immunohistochemistry¹⁵. However, we detected insignificant amounts of G1-IPEN³⁴¹ fragments in the OA synovial fluid with anti-G1 or anti-IPEN antibodies, while G1-TEGE³⁷³ was readily detected with both anti-G1 and anti-TEGE antibodies. Even though the sensitivity and affinity of the anti-TEGE and anti-IPEN neoepitope antibodies may differ, these results together with the results based on anti-G1 Western analysis show that there were insignificant amounts of G1-IPEN³⁴¹ fragments in the OA synovial fluid pool as compared to the OA cartilage pool (Fig. 5). This contrasts to other reports that have detected both -TEGE³⁷³ and -IPEN³⁴¹ neoepitopes in some individual OA synovial fluid samples, although the quantity of the -TEGE³⁷³ neoepitope was greater¹⁴.

There are at least two possible explanations for relative paucity of G1-IPEN³⁴¹ fragments in the OA synovial fluid, one not necessarily excluding the other. Firstly, after MMP cleavage at the -IPEN³⁴¹↓ 342 FFGV- site, which could have occurred either in the matrix with subsequent diffusion of the fragments into the joint cavity or within the joint cavity, the G1-IPEN³⁴¹ fragments were immediately degraded within the joint cavity preventing their detection. Secondly, it is possible that the majority of -IPEN³⁴¹↓ 342 FFGV-cleavage takes place within the pericellular or territorial matrix, with prompt internalization of the -IPEN³⁴¹ neoepitope by the

chondrocytes. Endocytosis of the -TEGE³⁷³ neoepitope has been shown to occur in a porcine cartilage explant system³⁷, and a similar process may occur with the -IPEN³⁴¹ neoepitope.

With regard to spatial localization of proteinase activities, it was shown by immunohistochemistry that even though both -TEGE³⁷³ and -IPEN³⁴¹ neoepitopes were present in OA cartilage, the -IPEN³⁴¹ neoepitope was reduced or absent from the most superficial parts of the articular cartilage, while the -TEGE³⁷³ neoepitope was present in the same location¹⁵. This may indicate that aggrecanase cleavage can be spatially distinct within the tissue from MMP cleavage. Early work suggested that in normal mature cartilage there are at least two metabolic pools of aggrecan – an active pool with short-lived aggrecan surrounding the chondrocytes and an inactive pool with long-lived aggrecan in the interterritorial matrix³⁹⁻⁴³. This is consistent with -TEGE³⁷³↓³⁷⁴ARGS- cleavage occurring in the interterritorial matrix, where fragment diffusion into the joint cavity may dominate, while the majority of cleavage at -IPEN³⁴¹↓³⁴²FFGV- takes place in the pericellular or territorial matrix where endocytosis may dominate. Such separate pathways have been proposed¹⁵, and are further supported by *in vitro* studies showing that aggrecanases are unable to cleave ³⁴²FFGV-fragments at the -TEGE³⁷³↓³⁷⁴ARGS- site³⁷.

The findings described here provide a basis for a model of aggrecan turnover in OA joint cartilage (Fig. 8). The pathway generating ³⁷⁴ARGS-fragments is initiated by aggrecanase action in the interterritorial matrix, generating aggrecan with variable C-terminal sequences, but still interacting with hyaluronan and link protein. These interterritorially located molecules are further cleaved by aggrecanase within the IGD, generating G1-TEGE³⁷³ fragments bound to hyaluronan and free unattached ³⁷⁴ARGS- fragments. An associated but yet little understood simultaneous degradation of hyaluronan may allow the release of G1-TEGE³⁷³ from the matrix^{11, 44}.

The pathway for generation of ³⁴²FFGV- fragments is initiated in the vicinity of metabolically active chondrocytes when newly synthesized aggregated proteoglycans are cleaved within the IGD by MMP, generating G1-IPEN³⁴¹ and ³⁴²FFGV-G3 fragments. G1-IPEN³⁴¹ fragments, bound to HA, are further processed by the chondrocytes. The ³⁴²FFGV-G3 fragments, bound within the matrix by interactions with the N- and C-terminal sequences, are cleaved in the CS2 region by aggrecanase generating ³⁴²FFGV- fragments with variable C-terminals. Finally, the model suggests that the different G3-truncated ³⁷⁴ARGS- and ³⁴²FFGV- fragments, together with some G1- fragments, are further processed and then diffuse into the joint cavity.

In summary, the results of the present study indicate that the degradation of aggrecan in human knee OA cartilage involves both MMPs and aggrecanases, albeit with an apparent dominant role for aggrecanases. Further examination of individual samples of human OA cartilage and synovial fluid with quantitative methods may provide information on individual variability relating to these pathways, as well as their relationship to age, disease stage and disease activity; information that may be relevant when considering therapies directed towards preventing cartilage matrix breakdown.

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Figure legends

- Fig. 1 Schematic illustration of human aggrecan with cleavage sites of aggrecanases (above) and matrix metalloproteinases (below). G1, G2 and G3 - globular domains 1-3; IGD – interglobular domain; KS – keratan sulfate enriched region; CS1 and CS2 – chondroitin sulfate enriched region one and two. Amino acid numberings are based on mature human aggrecan starting with the N-terminal sequence ¹VETS- (NCBI accession nr P16112). The cleavage sites represented by arrows were summarized from several publications (see text).
- Fig. 2 Western immuno-blot peptide blocking experiment against *in vitro* digested cartilage aggrecan. Human cartilage aggrecan (A1D1) digested *in vitro* with ADAMTS-4 (A; B; C; E lane-1 and lane-3) or with MMP-3 (D; E lane-2 and lane-4) was SDS-PAGE separated (3-8 % gels) and analyzed by Western immuno-blot in the presence or absence of synthetic peptides. (A) Anti-ARGS Western with 1.5 µg GAG per lane, +/- 10-µM neoepitope ARGSVILTVKGGC-peptide and +/- 10-µM spanning EGEARGSVILTVKPIF-peptide. (B) Anti-SELE Western with 1.25 µg GAG per lane, +/- 2-µM neoepitope CASTASELE-peptide and +/- 2-µM spanning EVVTASTASELEGRGT-peptide. (C) Anti-KEEE Western with 3.6 µg GAG per lane, +/- 2-µM neoepitope CEVAPTTFKEEE-peptide and +/- 2-µM spanning VAPTTFKEEEGLGS-peptide. (D) Anti-FFGV Western with 4.2 µg GAG/lane, +/- 10-µM neoepitope FFGVGGEEEDITVC-peptide and +/- 10-µM spanning DIPENFFGVGGEEEDC-peptide. (E) Anti-G1 Western with 4.2 µg GAG per lane, +/- 2-µM immunization CATEGQVRVNSIYQDKVSL-peptide. Mean values of

molecular weights of aggrecan fragments are given as kDa, and were calculated based on electrophoretic migration. The anti-G1A band, marked with # (Fig. 2E) was identified as the deglycosylation enzyme chondroitinase ABC (results not shown).

Fig. 3 Aggrecan fragments detected in MMP-3 and ADAMTS-4 *in vitro* digested A1D1-cartilage, and in OA synovial fluid (D1-fraction) by Western immuno-blot. The samples were SDS-PAGE separated (3-8 % gel) and analyzed by Western blot with different antibodies as shown in figure. Different amounts of GAG per lane were loaded to facilitate the comparison between the samples. The positions of molecular weight markers are indicated.

Fig. 4 Detection of high molecular weight aggrecan fragments in OA synovial fluid. Human OA synovial fluid fraction D1 was SDS-PAGE separated (3-8 % gel) and analyzed by Western immuno-blot with the following antibodies: (A) anti-G1, anti-SELE and anti-ARGS; (B) anti-KEEE, anti-FFGV and anti-SELE. Different amount of GAG per lane were loaded to facilitate comparisons. Mean values of molecular weights (in kDa) of aggrecan fragments were calculated based on electrophoretic migration.

Fig. 5 Detection of aggrecan fragment G1-TEGE and G1-IPEN by Western immuno-blot. The human samples were SDS-PAGE separated (4-12 % gel) and analyzed by Western immuno-blot using different antibodies as shown. Amount of protein loaded per lane as shown. Mean values of molecular weights of aggrecan fragments were calculated based on electrophoretic migration.

Fig. 6 High molecular weight aggrecan fragments detected in OA cartilage by Western immuno-blot. Human OA aggrecan cartilage-A1D1 was SDS-PAGE separated (3-8 % gel) and analyzed by Western immuno-blot with different antibodies as shown. Amount of GAG loaded per lane, the positions of molecular weight markers and the aggrecan fragments detected are shown.

Fig. 7 The main distribution of aggrecan fragments detected by Western immuno-blot in OA- cartilage (A1D1 and A1D3 samples) and OA synovial fluid (D1 and A1 samples). Fragments shown are generated by pathways initiated by aggrecanase action (A) and MMP-action (B) as shown in Fig. 8.

Fig. 8 A hypothetical model of the major proteolysis of cartilage aggrecan in OA joints. ARGS- and FFGV- fragments are generated by two different pathways (I and II). In the pericellular area, full length aggrecan monomers (G1-G2-G3) are expressed and aggregate with hyaluronan (HA) and link protein; cross-linking of the G3-domain with *e.g.* fibulin-2 may also occur. In pathway I, aggrecan turnover in the pericellular area starts by MMP cleavage within the IGD producing G1-IPEN and FFGV-G3 fragments. The HA-bound G1-IPEN fragments are endocytosed by the chondrocytes. The FFGV-G3 fragments are further processed in the CS2-region by aggrecanases, producing FFGV-fragments with various C-terminal sequences (FFGV-G2— ---), which are partly retained in the tissue by sequence interactions (\perp). In pathway II in the interterritorial area, aggrecan monomers are first cleaved in the CS2-region by aggrecanases (AGNASE) producing HA bound G1-fragments with various C-terminals. Then the G1-fragments are cleaved by aggrecanase within the IGD, generating HA-bound G1-TEGE fragments and unbound ARGS-fragments

having various C-terminals (ARGS-G2— ---). Finally, in the interterritorial matrix compartment the aggrecan fragments are further processed by aggrecanases and other proteases producing fragments that are released into the synovial cavity.

Table I

Aggrecan fragments detected by Western immuno-blot in samples from OA patients

Molecular weights of detected aggrecan fragments from electrophoresis as mean values in kDa.

---, Not detected; ND, Not determined

Aggrecan fragments	SF A1- fraction	SF D1- fraction	Cartilage A1D1- fraction	Cartilage A1D3- fraction	Cartilage A1D1-fraction <i>in vitro</i> digested with	
					ADAMTS-4	MMP-3
G1-IPEN ³⁴¹	52	---	---	52	---	51
G1-TEGE ³⁷³	65	---	---	65	64	---
³⁴² FFGV-G2-KS	80-95	---	---	---	---	77
³⁴² FFGV-G2-CS1	---	---	300	---	283	124, 166, 290
³⁴² FFGV-G2-SELE ¹⁵⁴⁵	---	310	---	---	312	---
³⁴² FFGV-G2-KEEE ¹⁷¹⁴	341	335	340	---	328	---
³⁴² FFGV-G2-G3	---	---	411	---	---	---
³⁷⁴ ARGS-G2-CS1	---	129-159	---	---	122-158	---
³⁷⁴ ARGS-G2-SELE ¹⁵⁴⁵	---	311	---	---	309	---
G1-G2-KS	ND	---	---	ND	---	134
G1-G2-CS1	---	200, 252	197, 217	ND	247	173, 213, 245
G1-G2-SELE ¹⁵⁴⁵	---	340	331	---	337	---
G1-G2-KEEE ¹⁷¹⁴	---	354	367	---	353	---
G1-G2-G3	---	---	429	---	---	---
¹⁹²⁰ LGQR-G3	---	103	---	---	104	---
¹⁵⁴⁶ GRGT-KEEE ¹⁷¹⁴	46	50	---	---	51	---

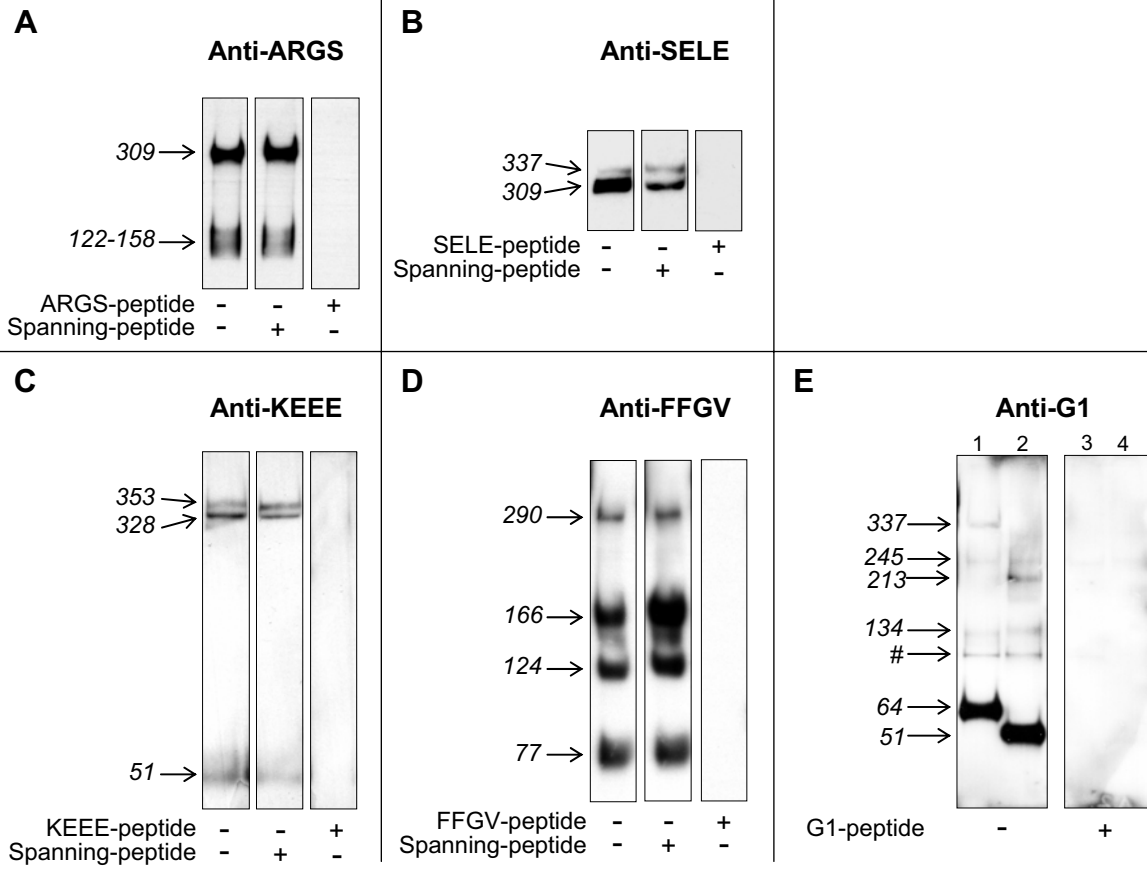
The superscript numbers denote the amino acid residue positions. Human aggrecan residue numbers used in this paper were obtained by subtraction of 19 amino acids (leader sequence) from the total (1-2415 amino acid) sequence of human aggrecan (NCBI accession nr P16112³).

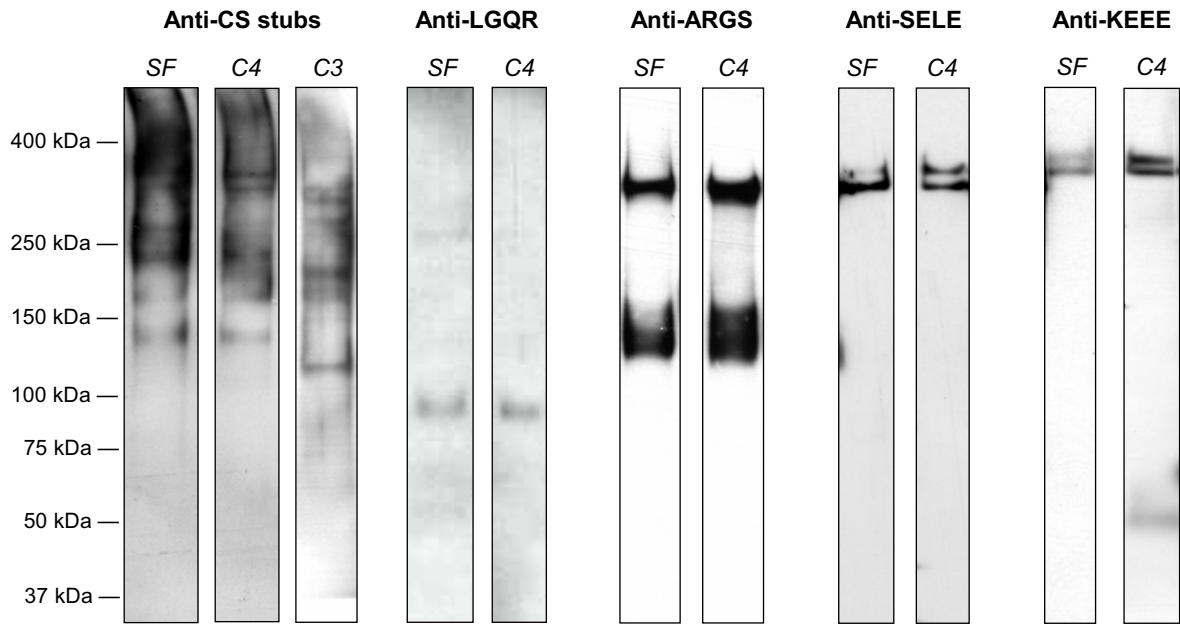
Table II

Calculated amounts of ARGS and FFGV fragments in synovial fluid from OA patients using Western immunoblot

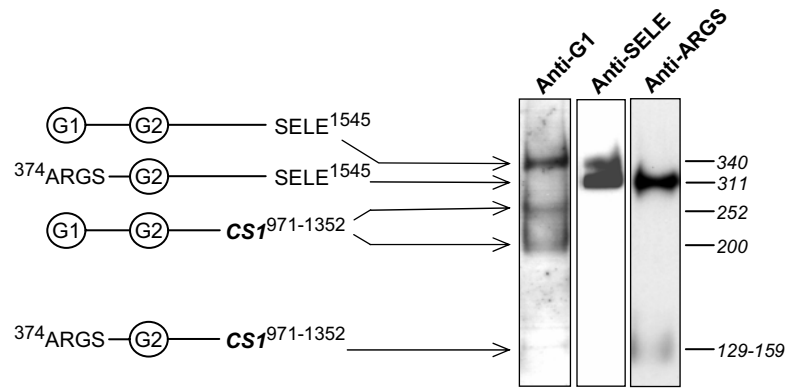
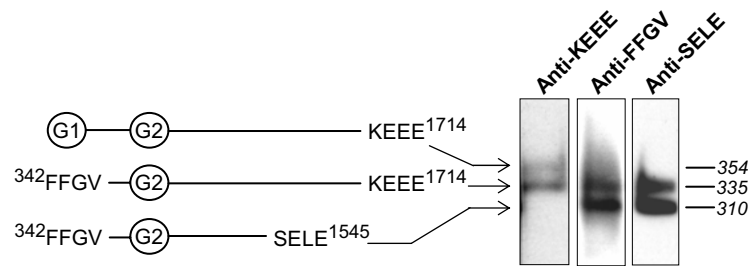
SF-D1 samples were probed with anti-ARGS or anti-FFGV antibodies. The samples were quantified using total ADAMTS-4 or MMP-3 cartilage-A1D1 digests as standards. Values are expressed as: amount of fragments per volume SF (neat synovial fluid) or amount fragments per mg GAG in the SF-D1 fraction. The data show total amounts of respective fragment, standard deviation values are shown in brackets for which n = 8.

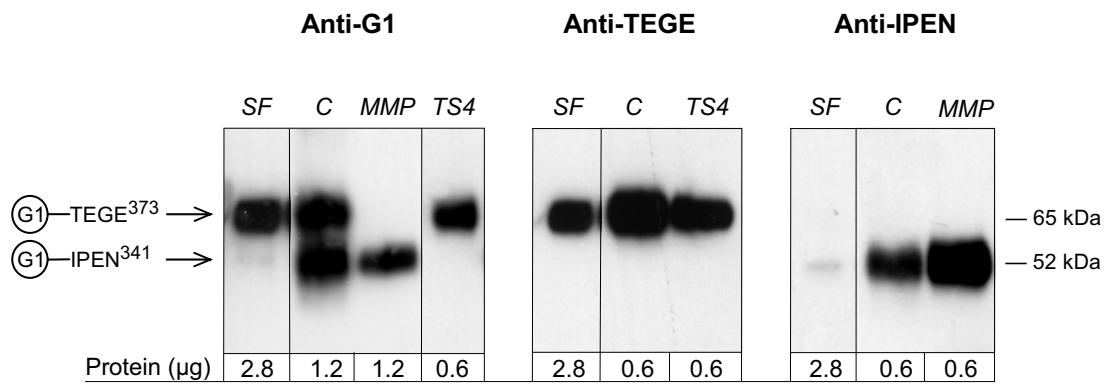
	Synovial fluid	
	pmol/ml SF	nmol/mg GAG
ARGS-fragments	107 (17.6)	1.13 (0.2)
FFGV-fragments	40.3 (9.4)	0.42 (0.1)
ARGS/FFGV (mol/mol)	2.7	
Total amount aggrecan	185 pmol/ml SF	





SF = Synovial fluid D1-fraction
 C4 = Cartilage-A1D1 *in vitro* digested with ADAMTS-4
 C3 = Cartilage-A1D1 *in vitro* digested with MMP-3

A**B**

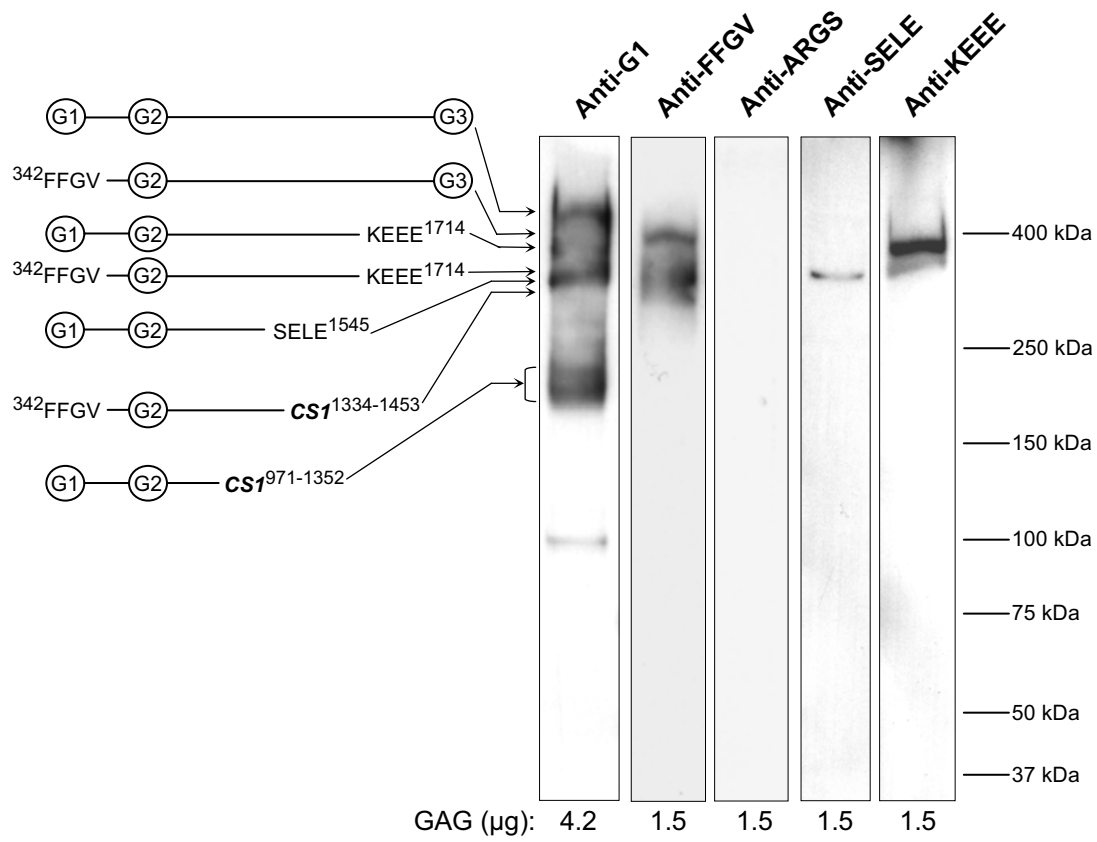


SF = Synovial fluid A1-fraction

C = Cartilage-A1D3

MMP = Cartilage-A1D1 *in vitro* digested with MMP-3

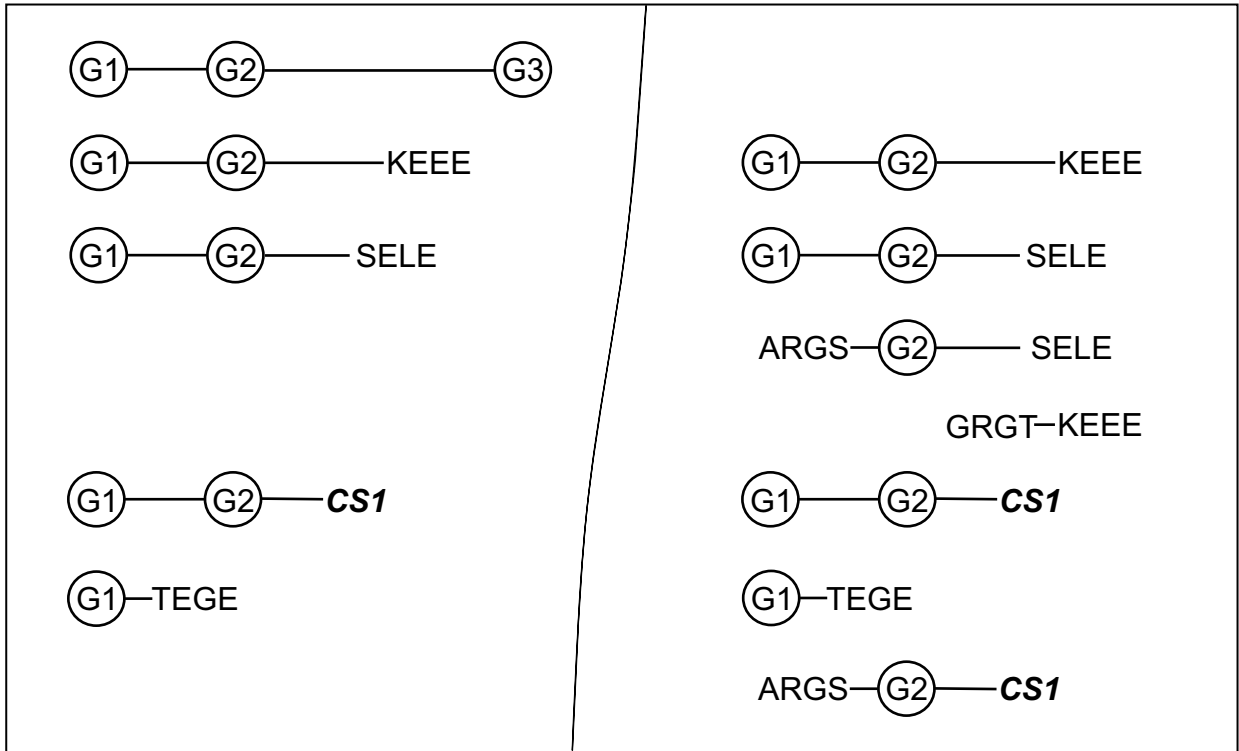
TS4 = Cartilage-A1D1 *in vitro* digested with ADAMTS-4



Cartilage

Synovial Fluid

A



B

