Elevated aggrecanase activity in a rat model of joint injury is attenuated by an aggrecanase specific inhibitor

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SUMMARY

Objective: To evaluate aggrecanase activity after traumatic knee injury in a rat model by measuring the level of aggrecanase-generated Ala-Arg-Gly-aggrecan (ARG-aggrecan) fragments in synovial fluid, and compare with ARG-aggrecan release into joint fluid following human knee injury. To evaluate the effect of small molecule inhibitors on induced aggrecanase activity in the rat model.

Method: An enzyme-linked immunosorbent assay (ELISA) was developed to measure ARG-aggrecan levels in animal and human joint fluids. A rat model of meniscal tear (MT)-induced joint instability was used to assess ARG-aggrecan release into joint fluid and the effects of aggrecanase inhibition. Synovial fluids were also obtained from patients with acute joint injury or osteoarthritis and assayed for ARG-aggrecan.

Results: Joint fluids from human patients after knee injury showed significantly enhanced levels of ARG-aggrecan compared to uninjured reference subjects. Similarly, synovial fluid ARG-aggrecan levels increased following surgically-induced joint instability in the rat MT model, which was significantly attenuated by orally dosing the animals with AGG-523, an aggrecanase specific inhibitor.

Conclusions: Aggrecanase-generated aggrecan fragments were rapidly released into human and rat joint fluids after injury to the knee and remained elevated over a prolonged period. Our findings in human and preclinical models strengthen the connection between aggrecanase activity in joints and knee injury and disease. The ability of a small molecule aggrecanase inhibitor to reduce the release of aggrecanase-generated aggrecan fragments into rat joints suggests that pharmacologic inhibition of aggrecanase activity in humans may be an effective treatment for slowing cartilage degradation following joint injury.

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Introduction

The destruction of articular joint cartilage is a hallmark of human arthritic diseases. Acute or chronic joint injury results in an imbalance between cartilage matrix synthesis and degradation. Cartilage breakdown following joint injury is reflected by an immediate significant increase in cleavage products of cartilage matrix proteins in the synovial fluid1,2. Concentrations of these molecular fragments remain elevated in the joint fluid for long periods but gradually decline over time. Degradative enzymes and breakdown products in the contra-lateral (CL) uninjured knee may also increase, suggesting that some responses to injury are systemic as well as local3. Elevated release of matrix proteins into the joint fluid has been shown also in animal models of joint disease or injury4,5. Increased expression and activity of catalytic enzymes such as aggrecanases and matrix metalloproteases (MMPs) result in the loss of aggrecan and collagen, respectively from the cartilage matrix6,7. Aggrecan cleavage at the E373–A374 [E392–A393, based on Accession numbers (Swiss-Prot:P16112) for human and (Swiss-Prot:P07897) for rat] bond of the interglobular domain (IGD) by aggrecanases results in the release of large sulfated glycosaminoglycan (sGAG)-substituted Ala-Arg-Gly-aggrecan (ARG-aggrecan) fragments which diffuse out of the cartilage matrix, and aggrecanase-generated aggrecan fragments have been identified in synovial fluids from osteoarthritic, joint injury, and inflammatory joint disease patients8–10.

In the current study we describe a novel assay to detect ARG-aggrecan fragments using commercially available reagents. Our results confirm and extend previous findings that ARG-aggrecan levels are increased in joint fluid of patients following traumatic

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injury, and further demonstrate a similar ARG-aggrecan release in the rat meniscal tear (MT) model. This animal model therefore serves to mimic human joint injury, enabling preclinical investigation of the role of post-traumatic aggrecanase activity. In addition, we show the potential for aggrecanase inhibition to prevent the release of aggrecanase-cleaved aggrecan into synovial fluid in the rat MT model.

**Methods**

**Human synovial fluid samples**

Human knee synovial fluids (n = 144) were obtained without lavage and were from Lund University, Sweden from a cross-sectional convenience cohort with informed patient consent and approval of the Lund University Ethics Committee. Diagnosis was made by arthroscopy, radiography, assessment of joint fluid and clinical examination. Diagnostic groups were knee healthy references (RF; n = 8), knee anterior/posterior cruciate ligament rupture, with or without concomitant meniscus lesions (ACL/PCL; n = 56), isolated knee meniscus injury (Mi; n = 69), and knee osteoarthritis (OA; n = 11). The mean OA grades of these groups based on Dahlberg et al.** were summarized in Table I. ARG-aggrecan was quantified in these samples at appropriate dilutions as described. Samples were tested at three different dilutions and results averaged for these assays.

**Aggrecanase inhibitor**

AGG-523 is a small molecule compound that was developed by structure-based design (US Patent Application Publication No. 2007/0043066), and is a reversible, non-hydroxamate, zinc-binding selective inhibitor of ADAMTS4 and ADAMTS5. The inhibition profile of the compound is summarized in Table II. The potency of AGG-523 to inhibit the enzymatic activity of metalloproteases or aggrecanases was tested in a continuous Fluorescent Resonance Energy Transfer (FRET) assay. The FRET assay uses specific substrates and protocols designed for individual enzymes. The Kᵢ (inhibitor dissociation constant) values for ADAMTS4 and ADAMTS5 were determined with a continuous fluorescence polarization (FP) assay.

**Rat MT model**

Male Lewis rats weighing approximately 300 g were obtained from Charles River Laboratories (Wilmington, MA). In the in vitro course study of ARG-aggrecan release, rats underwent medial MT surgery in the right knee to induce joint instability leading to cartilage degeneration as described. The animals were euthanized at different times after surgery (4 days–8 weeks, n = 10/time point). Synovial fluid lavages and serum were collected. Five naïve animals per time point were also included. In the study to determine the effect of specific aggrecanase inhibition on ARG-aggrecan release, animals (6–7 per group/time point) were dosed 1 week after medial MT surgery with three doses [twice a day (BID) dosing over 2 days] of vehicle [0.5% methyl cellulose/2% Polysorbate (Tweeze) 20] or 1000 mg/kg AGG-523. Synovial fluid lavages were collected from the knees at 2, 4, 6, or 8 h following the last dose. ARG-aggrecan levels were determined in synovial fluid lavage collected from surgery knees with CL joint fluids as controls. Serum and synovial fluid urea levels in each animal were used to correct ARG-aggrecan values for dilution. These studies were performed under the approval of Pfizer’s Institutional Animal Care and Use Committee.

**ARG-aggrecan enzyme-linked immunosorbent assay (ELISA)**

Arggrecan was prepared by extraction of sliced rat cartilage in 4 M Guanidine HCl, 50 mM sodium acetate pH 5.8 containing protease inhibitor followed by extensive dialysis. Arggrecan was cleaved with rhADAMTS4 prepared at Pfizer** (2.5 µg rhADAMTS4/mg aggrecan) in 50 mM Tris, pH 7.3, 100 mM NaCl, 5 mM CaCl₂, and deglycosylated with 0.0025 U chondroitinase ABC (Sigma, St Louis, MO), 0.0025 U Keratanase I and 0.00025 U Keratanase II (Seikagaku America, Fal-mouth, MA) per 10 µg sGAG (aggrecan) at 37°C for 3 h and used as a standard. Human aggrecan standard was prepared using protocols described with some modifications. Briefly, 1 g of human OA cartilage was pulverized under liquid nitrogen and suspended in 10 ml of 4 M guanidine HCl, 50 mM sodium acetate pH 5.8 containing 1× protease inhibitor cocktail and extracted for 48 h at 4°C. The mixture was then centrifuged at 3000 rpm for 10 min, supernatant separated on a CsCl gradient (100,000 g for 72 h at 4°C). The bottom 1/3 portion of the divided sample was dialyzed against Millipore water in the presence of 1× inhibitor and cleaved with rhADAMTS4 as described above. An estimated molecular weight of 1.5 × 10⁶ g/mol was used for aggrecan standard based on Struglics et al.** The concentration of rat and human aggrecan standards was expressed as the amount of proteoglycan measured prior to deglycosylation. Proteoglycan was determined by the sGAG assay (Kamiya Biomedical Company, Seattle, WA), a quantitative dye-binding assay for the in vitro analysis of sGAGs that uses chondroitin sulfate as the standard.

Monoclonal anti-human aggrecan antibody AHP0022 (Invitrogen, Carlsbad, California) that recognizes an epitope in both human and rat aggrecans was expressed as the amount of proteoglycan measured prior to deglycosylation. Proteoglycan was determined by the sGAG assay (Kamiya Biomedical Company, Seattle, WA), a quantitative dye-binding assay for the in vitro analysis of sGAGs that uses chondroitin sulfate as the standard.

**Table I**

ARG-aggrecan levels in human synovial fluids

<table>
<thead>
<tr>
<th>Study group</th>
<th>ACL/PCL (0–12 weeks)</th>
<th>ACL/PCL (12–52 weeks)</th>
<th>ACL/PCL (&gt;1 year)</th>
<th>MI (0–12 weeks)</th>
<th>MI (12–52 weeks)</th>
<th>MI (&gt;1 year)</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean sampling time (range)</td>
<td>1 (0–6)</td>
<td>25 (13–48)</td>
<td>279 (57–1115)</td>
<td>3 (0–12)</td>
<td>24 (13–40)</td>
<td>418 (52–1926)</td>
<td>189 (0–481)</td>
</tr>
<tr>
<td>No. of subjects</td>
<td>26</td>
<td>8</td>
<td>22</td>
<td>27</td>
<td>22</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Mean age (range in years)</td>
<td>25 (14–50)</td>
<td>31 (17–50)</td>
<td>31 (14–51)</td>
<td>34 (18–59)</td>
<td>35 (16–55)</td>
<td>45 (28–70)</td>
<td>67 (39–86)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1 (1–3)</td>
<td>2 (1–3)</td>
<td>3 (1–6)</td>
<td>2 (0–5)</td>
<td>2 (0–3)</td>
<td>3 (1–8)</td>
<td>8 (3–9)</td>
</tr>
<tr>
<td>% Male</td>
<td>62</td>
<td>63</td>
<td>68</td>
<td>93</td>
<td>86</td>
<td>70</td>
<td>45</td>
</tr>
<tr>
<td>Mean (range) ARG-aggrecan (µg/ml)</td>
<td>148 (19–588)</td>
<td>49 (9–195)</td>
<td>36 (2–107)</td>
<td>88 (7–394)</td>
<td>23 (2–94)</td>
<td>16 (2–85)</td>
<td>39 (6–133)</td>
</tr>
<tr>
<td>Mean confidence intervals for</td>
<td>95% (95–190)</td>
<td>95% (95–190)</td>
<td>95% (95–190)</td>
<td>95% (95–190)</td>
<td>95% (95–190)</td>
<td>95% (95–190)</td>
<td></td>
</tr>
<tr>
<td>Mean fold difference over Ref.</td>
<td>13.6</td>
<td>4.5</td>
<td>3.3</td>
<td>8.1</td>
<td>2.1</td>
<td>1.4</td>
<td>3.5</td>
</tr>
<tr>
<td>P value (compared to Ref.)</td>
<td>&lt;0.0001</td>
<td>0.0666</td>
<td>0.0575</td>
<td>0.0001</td>
<td>0.3683</td>
<td>0.9734</td>
<td>0.0461</td>
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</table>

Ref. = knee healthy references; ACL/PCL = knee anterior/posterior cruciate ligament rupture; Mi = knee meniscus injury; and OA = knee OA. Sampling time, age, OA grade and ARG-aggrecan are shown as averages with the range. Grades of 0–1 represent normal cartilage, 2–5 represent arthroscopic cartilage changes, and 6–10 represent radiographic changes. Mean ARG-aggrecan is the mean of averaged ARG-aggrecan values for subjects within a group tested in triplicates. P values are from a paired comparison based on the ANOVA of log transformed ARG-aggrecan values.
purified using a Protein-G antibody purification kit (Sigma). To test for specific binding of AHP0022 and BC-3 to human aggrecan and synovial fluid, 3–5 μg of human ARG-aggrecan standard (rhADAMTS4 digested human A1D1 fraction of cartilage) and a D1 fraction of synovial fluids pooled from OA patients 9 were deglycosylated, separated on a 3–8% Tris-acetate sodium dodecyl sulfate (SDS) gel, blotted onto a polyvinylidene fluoride (PVDF) membrane and probed with AHP0022, BC-3, or AGG-C119.

Table II

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC50 (μM)</th>
</tr>
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<tbody>
<tr>
<td>ADAMTS4</td>
<td>&lt;0.03 (K = 0.006)</td>
</tr>
<tr>
<td>ADAMT5S</td>
<td>0.04 (K = 0.1)</td>
</tr>
<tr>
<td>MMP-1</td>
<td>&gt;200</td>
</tr>
<tr>
<td>MMP-2</td>
<td>9.58</td>
</tr>
<tr>
<td>MMP-3</td>
<td>15% inhibition @ 25</td>
</tr>
<tr>
<td>MMP-7</td>
<td>4% inhibition @ 25</td>
</tr>
<tr>
<td>MMP-8</td>
<td>3.5</td>
</tr>
<tr>
<td>MMP-9</td>
<td>No inhibition @ 25</td>
</tr>
<tr>
<td>MMP-12</td>
<td>1.96</td>
</tr>
<tr>
<td>MMP-13</td>
<td>&gt;50</td>
</tr>
<tr>
<td>MMP-14</td>
<td>&gt;200</td>
</tr>
<tr>
<td>TACE</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

IC50s were determined by FRET assays using synthetic peptide for the respective enzyme containing a fluorescent group (7-methoxycoumarin or 2-aminobenzoyl) that was quenched by energy transfer to 2,4-dinitrophenyl. K, for ADAMT54 and ADAMT55 were determined by the FP assay. TACE-Tumor Necrosis Factor-alpha converting enzyme.

In order to determine spike-in recovery of aggrecan, two rat synovial fluids diluted at 1:1, 1:10, or 1:100 were spiked with 50 or 100 ng of ARG-aggrecan standard and analyzed in the ELISA. Within-assay precision for the ARG-ELISA was measured by assaying a pooled rat synovial fluid sample 96 times across the wells of a 96-well plate. Between-assay precision for the ARG-ELISA was determined by deglycosylating and assaying two rat synovial fluids four different times/days with respective standards. Similar experiments were also conducted with human synovial fluids for the technical validation of human sample detection.

**ARG-aggrecan quantitation using LC-MS/MS**

A multidimensional (immunoafluorimmunoassay/reversed-phase) LC-MS/MS analysis of ARG-aggrecan in rat synovial fluid lavage was done for specific samples following protocols described22. Briefly, a representative peptide (ARGNVL) was created by digestion with chymotrypsin and quantified as a measure of aggrecan in synovial fluid. Although the specificity of chymotrypsin is relatively broad23,24, the ability to cleave on the C-terminal side of aromatic and large hydrophobic residues was exploited to reproducibly cleave aggrecan after leucine residues and produce peptides suitable for LC-MS/MS. A slightly longer deuterated internal standard peptide ARGN(d8)WLTAK was added to correct for both digestion efficiency and injection volumes. Both the heavy and light forms of the peptide, ARGNVIL were quantitatively detected by monitoring high-performance liquid chromatography (HPLC) elution times and ion pairs corresponding to the specific multiple reaction monitoring (MRM) pairs (371.5/470.3). The digested synovial fluid samples were enriched on an antibody column (BC-3) and analyzed by LC-MS/MS. Peptide abundances were determined by comparing the LC-MS/MS peak areas of the analytes to those of deuterated internal standards. Standard curves were prepared at concentrations ranging from 1 to 1000 pg/ml (0.001–0.135 pmol/ml based on the MW of 741 Da for the peptide).

**Statistical analysis**

One-way Analysis of Variance (ANOVA) of log transformed ARG-aggrecan values from human and rat synovial fluids was performed to test for statistical significance. Spearman’s rank order was used for correlation analysis.

**Results**

**ARG-aggrecan ELISA**

ADAMTS4 digested and deglycosylated aggrecan fragments bound by anti-aggrecan antibody AHP0022 in the 96-well
format sandwich ELISA were detected by antibody BC-3, which recognizes only ARG-positive aggrecan generated from specific aggrecanase cleavage in the IGD of aggrecan. Absorbance values from the standards at a range of 0.078–5.0 mg of aggrecan (glycosaminoglycan)/ml were fitted to a 4-parameter logistic model ($R^2 = 1.0$). The range of detection for the assay was 0.1–5.0 μg/ml (or 0.067–3.333 pmol/ml) aggrecan. The output signal was concentration dependent up to 5 μg/ml total proteoglycan.

**Specificity of the ELISA method**

In the Western analysis of human aggrecan standard and synovial fluid, BC-3 and AHP0022 recognized a 310 kD ARG5-G2-SELE band [Fig. 1(A); species 1], and 120–160 kD ARG5-G2-CS1 bands [Fig. 1(A); species 2 and 3] similar to already reported ARG5-aggrecan fragments. An additional 69 kD band, reactive with both BC-3 and AHP0022, was detected in purified synovial fluid [Fig. 1(A); species 5]; plausibly an ARG5-G2-GVA fragment based on its reactivity with
an antibody specific for the calpain generated GVA C-terminus (not shown)\textsuperscript{25}. The G1-TEGE fragments generated by ADAMTS4 digestion of the standard were reactive with both AGG-C1 (anti-TEGE)\textsuperscript{19}, and AHP0022 [Fig. 1(A); species 4]. This data indicates that AHP0022 is binding to G1 and/or G2-positive aggrecan, and that it has the capability of capturing all ARG5-positive aggrecan found in the standard and in this purified synovial fluid.

The ELISA detected ADAMTS4-cleaved rat/human aggrecan but not intact aggrecan or MMP-13 cleaved aggrecan confirming the specificity of the assay [rat aggrecan data shown in Fig. 1(B)]. Deglycosylated standards (0.078–5.0 μg/ml) plated on PBS- or mouse IgG-coated wells served as negative controls and did not produce any optical density (OD) values in the ELISA confirming specific binding of aggrecan to AHP0022-coated plates. Non-deglycosylated rat/human synovial fluids produced insignificant or no absorbance values. The binding of BC-3 to aggrecan required deglycosylation, whereas AHP0022 bound to both glycosylated and deglycosylated forms of aggrecan. This was determined in a direct binding assay where human aggrecan standard (deglycosylated or non-deglycosylated) was coated onto a plate followed by incubation of BC-3 or AHP0022, addition of HRP-conjugated anti-mouse secondary antibody, and detection. This was further evidenced by a recent publication\textsuperscript{26}. The binding of AHP0022 to the core protein was confirmed by testing the ability of the antibody to bind to recombinant human aggrecan G1-IGD-G2 (R&D Systems, Minneapolis, MN) in the ELISA (data not shown). When aggrecan in synovial fluids was depleted with the anti-aggrecan antibody, AHP0022 or AHP0012, or using DEAE-650S, 100% loss of ARG5-aggrecan signal was observed in the assay confirming the specificity of detection.

Technical validation of the ELISA

The mean spike recovery of rat aggrecan at dilutions of 1:5, 1:10, and 1:20 was 92% with a range of 82–96% for rat synovial fluids tested. The mean spike recovery of human aggrecan at dilutions of 1:50, 1:100, and 1:200 was 102% with a range of 74–134% for human synovial fluids tested. The coefficient of variation (CV) for within-assay precision measured by assaying a rat or a human synovial fluid 96 times across the same plate was 2.4%. The between-assay precision for rat samples was measured by assaying two rat joint fluids four different times with respective standards on separate days including independent sampling and deglycosylation. The CV of two samples was determined to be 3.5% and 3.3%, respectively. The between-assay precision for human samples was similarly determined by assaying four human joint fluids; the CVs were 1.7, 1.7, 2.5, and 2.0%, respectively.

ARG5-aggrecan ELISA vs LC-MS/MS

Synovial fluid lavage samples ($n = 28$) from a 1-week-rat MT study tested in the ELISA were also analyzed in the LC-MS/MS assay. ELISA data measured as μg/ml ARG5-aggrecan was converted to pmol/ml assuming an average aggrecan molecular weight of 1.5 × 10\textsuperscript{6} Da. Similarly, LC/MS-MS data quantified as ng/ml analyte was converted to pmol/ml based on the molecular weight of 741 Da for the ARGNVIL standard peptide. A significant correlation was observed between measured ARG5-aggrecan levels in these two assays as determined by the Spearman’s rank order correlation ($r_s = 0.95$; Fig. 2) confirming the specificity of detection in the ELISA method.

ARG5-aggrecan in the joint fluid of human patients with knee injury/disease

ARG5-aggrecan in human synovial fluids showed a wide range of concentrations from 2 to 588 μg/ml. Significantly higher levels of ARG5-aggrecan compared to reference subjects were observed in ACL/PCL 0–12 weeks post-injury, MI 0–12 weeks post-injury, and OA synovial fluids. The observed increases in the ARG5-aggrecan levels of ACL/PCL and MI patients compared to reference levels decreased over time following joint injury (Table I).

ARG5-aggrecan in rat synovial fluids and effect of aggrecanase inhibition

There was a significant increase in ARG5-aggrecan levels in operated unstable right knees (4-fold) 4 days and 1 week after surgery as compared to un-operated CL left knees or to naive animals (left and right knees). The significant increase in ARG5-aggrecan levels in unstable knees compared to CL knees or naive animals was maintained up to 8 weeks post-surgery (Fig. 3).

When rats were treated with three doses of 1000 mg AGG-523/ kg BID, starting a week after surgery, and synovial fluid lavage collected 2, 4, 6, or 8 h after last dosing, there was complete inhibition of surgery-induced ARG5-aggrecan release in unstable knees of the inhibitor-treated rats as compared to vehicle-treated rats at 6 and 8 h after last dosing of AGG-523 (Table III). ARG5-aggrecan levels in the surgery knees of AGG-523 treated rats at these time points were close to or lower than levels observed in the un-operated CL knees of the vehicle-treated animals. The inhibitions at 2 and 4 h after last dose were 38% and 78%, respectively, considering a 100% inhibition for levels similar to ARG5-aggrecan in un-operated knees of vehicle-treated animals. There was a similar reduction in the basal level of ARG5-aggrecan in un-operated CL knees with AGG-523 treatment (Table III).

Discussion

A novel ELISA was developed to quantify aggrecanase-generated ARG5-aggrecan fragments in both human and rat synovial fluids. Results from the assay of human samples indicate that aggrecanase activity was increased significantly in the period immediately after injury, and, although joint fluid levels of ARG5-aggrecan decline over time, they were significantly higher than normal levels up to 12 weeks after injury, and showed a trend toward persistence of raised levels thereafter which was not statistically significant. The levels of ARG5-aggrecan in OA patients were similar to the measured levels in ACL/PCL patients > 1 year post-injury (Table I). The same time-related trends of ARG5-aggrecan release following injury were seen in the rat model, and the capacity to inhibit aggrecanase-cleaved aggrecan release with a small molecule aggrecanase inhibitor was demonstrated.
The first quantitative assay to determine ARG-aggrecan concentrations in biological samples was reported by Pratta et al. using a sandwich anti-KS/mAb OA-1 ELISA with proprietary reagents. This assay was used in a recent cross-sectional study of human joint fluids. However, as discussed by Larsson et al., a limitation to their ELISA format using anti-keratan sulfate antibody for capture could arise from variability in the degree of keratan sulfate substitution in the aggrecan population.

A similar assay format has been used as one of the tools to analyze samples from a rat monosodium iodoacetate (MIA) model but this study does not include assay development, validation steps and assay qualification. Consequently, a newer method was described by the same group using BC-3-C2 (optimized BC-3), a proprietary antibody for quantifying ARG-aggrecan fragments in synovial fluid, serum and urine. The ELISA method in our study that has been developed, validated, clinically qualified, and specificity confirmed using an orthogonal assay format is, however, the first of its kind that detects ARG-aggrecan fragments in biological samples using commercially available reagents. Our method is also sensitive and suitable for testing synovial fluids spanning a wide range of ARG-aggrecan concentrations (lower limit of approximately 0.1 mg/ml for rat synovial fluid and 1 mg/ml for human synovial fluid).

Four assumptions have been necessary in the quantification of ARG-aggrecan by ELISA in synovial fluids: (1) Digestion of aggrecan with rhADAMTS4 rendered a complete conversion of aggrecan with an intact IGD into C-terminal ARG-aggrecan and N-terminal TEGE-aggrecan fragments. (2) The proportion of differently sized aggrecan fragments detected in synovial fluid equals those found in the standards. (3) All ARG-aggrecan fragments also contain the G2 domain. (4) All the aggrecan fragments containing both ARG and G2 have the same molar reactivity with BC-3 and AHP0022, irrespective of size, GAG content, and C-terminal truncation.

Binding of AHP0022 to all of the ARG-aggrecan species in human aggrecan standard and purified synovial fluid detected by

![Fig. 3. Time course of ARG-aggrecan release in the rat MT model. Rats were euthanized at different times after surgery (4 days–8 weeks, n = 10 rats/time point). Five naive rats (10 joints) per time point were also included in the study. For each time point, 10 synovial fluid lavage samples from the right MT, left CL, and naive (N; left + right knees) joints were tested in the ARG-aggrecan ELISA and values corrected for dilution using serum urea levels. Average ARG-aggrecan with the error bars indicating 95% confidence intervals. No correction was made in statistical testing for naive right-left knees not being independent. P values are included in the graph.](image)

### Table III

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Right unstable knee</th>
<th>Left CL knee</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000 mg/kg AGG-523</td>
<td></td>
</tr>
<tr>
<td>2 h</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>4 h</td>
<td>10.1</td>
<td>5.7</td>
</tr>
<tr>
<td>6 h</td>
<td>16.8</td>
<td>9.5</td>
</tr>
<tr>
<td>8 h</td>
<td>23.9</td>
<td>11.6</td>
</tr>
</tbody>
</table>

Three oral doses of vehicle or 1000 mg/kg AGG-523 were administered over 2 days and synovial fluid harvested at the indicated time points post last dose. Of the 7 vehicle rats, synovial fluid harvested at 6 h after last dosing from 4 rats, and at 8 h after last dosing from 3 rats. Right knees of animals underwent surgery; left non-surgery knees served as controls. P values are from a paired comparison based on the ANOVA of log transformed ARG-aggrecan values.
BC-3 [Fig. 1(A)] indicates that all predominant ARG-fragments in human aggrecan standard and synovial fluid are captured by AHP0022 in our assay format. However, two minor MMP cleavage sites (from in vitro digestion with excess enzyme) in the IGD of aggrecan have been reported: 384-D-V and 444-D-L, which might result in the loss of G2 domain from ARG-aggrecan fragments that are recognized by AHP0022 in the assay. Even though G1 fragments reflecting cleavage at these sites have not been reported in human aggrecan preparation cleaved by MMP in vitro or in human synovial fluids tested, loss of G2 domain is possible from some AGP-aggrecan fragments due to multiple MMP cleavages.

Detection of specific fragments in the LC-MS/MS method and the significant correlation of ARG-fragments quantified in these two assays (Fig. 2) not only confirm specificity of detection in the ELISA but also suggest that the ELISA method captures and quantifies all AGP-aggrecan species similar to a method that involves direct binding of BC-3 to AGP-positive aggrecan fragments. However, the discrepancy seen between the ELISA and LC-MS/MS assays could arise from the different standards (aggrecan purified from cartilage with a roughly estimated MW vs specific synthetic peptide) used in these assays.

The major advantages of the present method are that it uses commercially available reagents and that the assay is applicable to testing synovial fluid from animal joints in addition to human joints. Homologous aggrecanase cleavage sites and cleavage products have been reported in bovine, equine, porcine, and murine aggrecan. The applicability of this assay to detect aggrecan from various species makes it a good candidate to analyze samples from preclinical animal models. We have detected AGP-aggrecan fragments in the synovial fluid of non-human primates and guinea pigs in addition to humans and rats (data not shown).

Transection of the medial meniscus in rats leads to histological changes consistent with OA as rapidly as 1–2 weeks after surgery, progressing over time to severe cartilage degeneration. The cartilage lesions that occur in this joint instability model are morphologically similar to those that occur in human OA, although the rate of development of damage is greatly increased in rats compared to humans. Induction of OA in rats by surgical tear of the meniscus thus serves as a relevant animal model representing joint disease as calpain generated ARG-EDLS fragments have recently been identified specifically in the synovial fluid of patients with acute inflammatory arthritis. We have identified a calpain generated ARGS-G2-GVA species in the synovial fluid pool from OA patients [Fig. 1(A)]. Detailed studies are ongoing to better understand the rat MT model in terms of formation and clearance of ARGP-aggrecan in the joint.

GAG content was replenished at early stages of aggregcanase-mediated aggrecan damage, whereas the loss of structural integrity was irreversible after collagen damage at later stages of explant cultures. In addition to its functional role in cartilage load-bearing, aggrecan may have a protective role in preventing type II collagen loss from the cartilage matrix. Indeed, reduction of aggrecan catabolism has been shown to abrogate the development of experimental arthritis in ADAMTS5 knockout mice. Blocking cleavage at the IGD in the knock-in mice diminished aggrecan loss and cartilage erosion in the OA model. However, only the spread of the OA-like pathology in the cartilage was curtailed but the focal cartilage damage was not prevented in the knock-ins. Furthermore, ADAMTS4 and ADAMTS5 knockout mice and double knockout mice are viable, fertile, and demonstrate no gross abnormalities. Together, these results warrant further studies of aggregcanase inhibition as a means of decreasing cartilage damage in joint disease.

Extensive literature documents the relationship between knee injury and subsequent OA in humans. Young adults with acute knee injury have an accelerated risk of developing symptomatic OA and thus represent an attractive population for disease modifying interventional studies. Longer term studies of aggregcanase activity and joint changes in these patients might provide insights as to how the degradative mechanisms that initiate with traumatic insult to the knee translate into a progressive disease.

Authors’ contributions

PSC was primarily responsible for the conception and design of the ELISA, assay development, analysis & interpretation of data, and preparation of the manuscript. WS generated and analyzed most of the ELISA data. WZ contributed to data acquisition and analysis. DRD designed the LC-MS/MS assay. MARB and SSG were responsible for the design and data interpretation of animal models. LSL and SL participated in the collection of human samples, and provided clinical, biomarker, and Western analysis data, respectively, and revised the manuscript critically for important intellectual content. CRF contributed to the conception of the study, provided critical input on the ELISA design and contributed to data interpretation and final design/approval of the manuscript. KEG contributed to the design and development of AGG-523 and participated in the design and data interpretation of animal studies. EAM contributed to the conception and design of the study and sample acquisition, data interpretation and design/approval of the manuscript.

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Conflict of interest

PSC, WS, MARB, WZ, DRD, CRF, SSG, KEG, and EAM are current or past employees of Pfizer and hold company stocks/stock options. SL and LSL have no competing interests.

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