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# Complement 1s is the Serine Protease that Cleaves IGFBP-5 in Human Osteoarthritic Joint Fluid

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## Abstract

Insulin-like growth factor-I (IGF-I) and IGF binding proteins (IGFBPs) are trophic factors for cartilage and have been shown to be chondroprotective in animal models of osteoarthritis. IGFBP-5 is degraded in joint fluid and inhibition of IGFBP-5 degradation has been shown to enhance the trophic effects of IGF-I.

**Objective—**To determine the identity of IGFBP-5 protease activity in human osteoarthritic (OA) joint fluid.

**Method—**OA joint fluid was purified and the purified material analyzed by IGFBP-5 zymography.

**Results—**Both crude joint fluid and purified material contained a single band of proteolytic activity that cleaved IGFBP-5. Immunoblotting of joint fluid for complement 1s (C1s) showed a band that had the same Mr estimate, e.g. 88 kDa. In gel tryptic digestion and subsequent peptide analysis by LC-MS/MS showed that the band contained human complement 1s. A panel of protease inhibitors was tested for their ability to inhibit IGFBP-5 cleavage by the purified protease. Three serine protease inhibitors, FUT175 and CP 143217 and CB-349547 had IC50's between 1and 6 uM. Two other serine protease inhibitors had intermediate activity (e.g. IC50's 20–40 uM) and MMP inhibitors had no detectible activity at concentrations up to 300 uM.

**Conclusion—**Human OA fluid contains a serine protease that cleaves IGFBP-5. Zymography, immunoblotting and LCMS/MS analysis indicate that complement 1s is the protease that accounts for this activity.

### Keywords

Insulin-like growth factor I; Insulin-like growth factor binding protein-5; complement 1s; chondrocyte

## Introduction

IGF-I is a potent stimulant of chrondrocyte extracellular matrix protein synthesis and growth(1,2). Following its synthesis in liver IGF-I is transported to target tissues, such as cartilage, where it stimulates growth (3). IGF-I is also synthesized by cartilage and this locally synthesized IGF-I stimulates epiphyseal growth (4). Direct injection of GH into the

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growth plate of hyposphysectomized animals stimulates IGF-I synthesis and cartilage growth. Simultaneous administration of an IGF-I antibody results in attenuation of the cartilage growth response (5). In mice deletion of hepatic IGF-I gene expression reduces blood IGF-I concentrations by 80% but has a minimal effect on statural growth (6% reduction) whereas if IGF-I synthesis in cartilage and other tissues is eliminated growth is attenuated by 50% (6,7). In addition, growth plate chondrocytes in the proliferative zone possess abundant IGF-I receptors and both growth plate and articular chondrocytes respond to IGF-I in vitro with increases in DNA and proteoglycan synthesis (2,8). Together, these findings support the conclusion that locally produced IGF-I is an important cartilage growth factor.

Treatment of canine osteoarthritis with IGF-I results in articular cartilage preservation and exposure to other cartilage growth factors enhances the cartilage response to IGF-I (9,10). In human osteoarthritis there is upregulation of IGF-I synthesis (11). There is also increased IGF-I synthesis in the synovium of inflamed joints and IGF-I augments chondrocyte proliferation after in vivo injury (12,13). Furthermore adenoviral mediated gene transfer of IGF-I into joints has been shown to have a protective function for articular chondrocytes in animal models of arthritis (14–17).

IGF binding proteins are synthesized by articular cartilage both during normal growth and during repair after injury (12) (18–20). Both IGFBP-3 and 5 have been shown to be upregulated during the early phases of articular chondrocyte differentiation and then downregulated when the cells become hypertrophic (18). Upregulation of IGFBP-5 was shown to be associated with enhanced IGF-I activation of the PI-3 kinase pathway in growth plate chondrocytes (21). In osteoarthritic articular cartilage, there is enhanced expression of IGFBP-3, 4 and 5 (20). The ratio between IGF-I and IGF binding proteins appears to be important since disruption of the IGFBP-3/IGF-I complex has been shown to enhance IGF-I actions (22), however IGFBPs also perform an important storage function in the joint and if all binding activity is eliminated IGF-I is a less effective growth stimulant. IGFBP-3 is abundant on the surface of articular chondrocytes and in osteoarthritic joints and has been reported to make the cells refractory to IGF-I (23). However in some studies IGFBP-5 enhanced both growth plate and articular chondrocyte proliferation (21,24,25). One variable that regulates IGFBP-5 is proteolysis and IGFBP-5 protease activity is increased in joint fluid during the development of arthritis (24–27). Inhibition IGFBP-5 cleavage was shown to limit the amount of articular cartilage destruction in dogs during the development of osteoarthritis. This was associated with an increase in the amount of IGF-I in joint fluid as well as an increase in intact IGFBP-5 (24). These findings suggest that in certain situations IGFBP-5 can act as a reservoir for IGFs in cartilage and synovial fluid and that factors that regulate rate of IGFBP-5 cleavage may alter the ability of this tissue to respond to IGF-I. Several proteases have been shown to cleave IGFBP-5 (28) however only complement 1s (24) and HTRA-1 (30,31) have been shown to be increased in osteoarthritis. These studies were undertaken to characterize the type of proteolytic activity that is present in human osteoarthritic joint fluid and to determine the specific protease that accounts for this activity.

## Methods

### Purification of protease activity

50 cc of knee joint fluid was obtained from patients with active osteoarthritis. This was defined using the criteria of the American College of Rheumatology. The patients were undergoing arthroscopy or knee replacement. All patients gave informed consent. 50 ml of joint fluid was added to 450 ml of 0.05 M Tris, pH 7.2 containing 1 M of ammonium sulfate and stored for 14 hr at 4°C then centrifuged 23,000 × g for 20 min. The supernatant was loaded onto a 4.4 × 3.5 cm butyl sepharose column-4 (Fast Flow Pharmacia Biotech) that

had been equilibrated with 0.07M Tris, pH 7.2, 1 M ammonium sulfate. The column was washed with 2 column volumes of the equilibration buffer and eluted with 0.025 M, Tris, pH 7.2 then the fractions were analyzed for IGFB-5 proteolytic activity. The active fractions were pooled then loaded onto a wheat germ agglutinin affinity column (1.6 × 5.0 cm) (Sigma) equilibrated 0.025 M Tris, 4 mM $CaCL_2$, 0.4 M NaCL pH 7.2. After washing (2 column volumes) the activity was eluted with 0.5 M N-acetyl-D-glucosamine. The fractions which had the greatest activity were pooled, diluted 1:2.5 with 25 mM Tris, pH 7.2, 4 mM CaCl2, 2mM Mn $CL_2$, 0.4 M NaCL then loaded onto a concanavalin-A agarose (Con-A) column (1 × 9 cm) (Sigma). After washing (5 column volumes) the activity fractions was eluted with 0.25 M, methyl α D, glycopyranoside and 0.25 M methyl α D mannopyranoside. The fractions with proteolytic activity were pooled prior to SDS-PAGE separation.

## Assay of IGFBP-5 proteolytic activity and immunoblotting of C1s

10 ul of each fraction was incubated with 0.05 ml of 0.05 M Tris, 4mM $CaCL_2$, pH 7.2, for 16 hr at 37°C containing 118 ng of pure human IGFBP-5 (32). The incubation the reaction was terminated by adding 50 ul of 4 × Lammeli sample buffer. The amounts of intact and cleaved IGFBP-5 were determined using SDS-PAGE followed by immunoblotting. 50 ul of the mixture was electrophoresed through a 12.5% gel and transferred to an Immobilon filter. The filters were probed using a 1:2000 dilution of a rabbit polyclonal antiserum (32). The immune complexes were detected using enhanced chemiluminescense (33). The fractions that contained proteolytic activity were analyzed by immunoblotting for C1s using a 1:1000 dilution of rabbit anti C1s antiserum (28).

To determine the effect of purified protease inhibitors, a mixture of 0.05 ml of 0.05 M Tris, 4 mM $CaCL_2$, pH 7.2, 118 ng of IGFBP-5 and inhibitor concentrations between 0.2 and 300 uM were incubated for 16 hours at 37°C. Either 1.6 ml of crude joint fluid or 2 ul of material that had been purified as described previously was used as a source of the protease. The incubation was terminated by adding 45 ul of 4× Laemmli sample buffer and the samples were immunoblotted for IGFBP-5 as described above. The IGFBP-5 antibody detects both intact IGFBP-5 and the major 22 kDa fragment (32). The immunoblots were developed using a goat antirabbit IgG alkaline phosphatase conjugate and chemiluminesence. Proteins were detected using enhanced chemilumiescense supersignal, CL-HRP system (Pierce, Rockford, Ill) and Kodak XAR film. Scanning densitometry was performed using an AGFA Scanner (Brussels, Belgium) and the results were analyzed using NIH Image version 1.61. To calculate the IC50 for each inhibitor the results of at least 2 separate experiments were analyzed

Some chromatographic fractions were also immunoblotted for HTRA-1 using rabbit polyclonal antiserum that was prepared using two synthetic HTRA-1 peptides as described previously (29).

## Joint fluid zymography

10 mcg of IGFBP-5 was mixed with 4 ml of acrylamide gel solution (10% gel) and the gel was polymerized. The samples of synovial fluid (1.6 ul) or Con-A column eluate (12 ug) were electrophoresed at 22°C. After washing in 2.5% TritonX-100 at 4°C for 1 hr the gel was incubated overnight in 0.05 M Tris, 4 mM $CaCl_2$ pH 7.4 at 37°C to allow for proteolysis and capillary transfer of the IGFBP-5 fragments to a PVDF membrane. The membrane was immunoblotted using a 1:2000 dilution of IGFBP-5 antiserum as described above. The electrophoretic mobility of the bands that were detected was compared with the prestained molecular weight standards (Life Technologies).

## Protein Identification by Mass Spectrometry

The material that had been purified through the Con-A affinity chromatography step (250 ul) was concentrated and the buffer exchanged into 2 × Lammeli sample buffer using an Ultrafree 0.5 ml centrifugal filter Biomax 10k/MWL (Millipore). 250 ug of protein in 50 ul was loaded; the proteins were separated by SDS-PAGE, 7.5% gel. The gel was stained with 0.1% Coumassie blue R250 (Sigma) in 10% acetic acid then destained in 50% methanol for 2hrs. The bands with mass estimates of 88 and 92 kDa were excised, washed 3× in 0.2 M Tris pH 8.0/50% acetonitrite then incubated in 250 ul of 0.1 M Tris, pH 8.0 with 2.0 ug trypsin and 4 mM DTT for 14 hr at 37°C. Following sonication (30 min RT) 1.0 ug of trypsin was added and the incubation continued for 5 hr at 37°C. The peptides were extracted in 100 ul of 0.1 M Tris, pH 8. This was repeated using 400 mcl of buffer followed by two extractions with 400 mcl of 60% acetonitrile, 0.2% TFA. The 4 extracts were pooled and concentrated 4 ×.

The samples were the analyzed on a Q-TOF (quadrapole time-of flight) mass spectrometer (Waters, Beverly, MA, USA) coupled to a CapLC system (Waters, Milford, MA, USA). 49 μL of sample was injected via “microliter pickup” mode and desalted and concentrated on-line through a peptide CapTrap cartridge (Microm BioResources, Auburn, CA). The samples were desalted at high flow (40μL/min). The peptides were separated on a Pepmap column (75 μm × 150 mm, 3 μm particle size) (LC packings, San Francisco, CA, USA). Separation was achieved using a 180 minute step gradient of 95% water, 0.1% formic acid to 75% acetonitrile, 0.31% formic acid. The flow rate was increased linearly with organic mobile phase from 250 to 400nL/min.

Data dependent MS/MS experiments were performed using a modified nanospray source designed to hold a distally coated picotip (360 μm OD × 20 μm ID × 10 μm tip diameter) (New Objective, Cambridge, MA). The instrument parameters were: capillary voltage, 1.4 to 1.6 kV; cone voltage, 35V, and source block temperature 140°C. The collision energy was determined in real time based on the mass and charge state of the peptide. Charge state recognition was used to switch into MS/MS mode on all doubly, triply, and quadruply charged ions above a certain threshold. The mass spectrometer monitored up to three components per survey scan. Spectra were acquired in MS mode at 1 second/scan and MS/MS mode at 2 seconds/scan. The MS switched back from MS/MS to MS based on either of two criteria: 6 seconds or maximum intensity of 3000. Major trypsin autolysis ions were excluded. Tuning and calibration of the Q-TOF mass spectrometer were performed while infusing 1 pmol/μL porcine somatotropin (PST) tryptic digest into the mass spectrometer. The data was processed by ProteinLynx version 3.5 (Waters, Beverly, MA) to generate searchable .pkl files. These files were searched using the search engine MASCOT (Matrix Science, Ltd).

## Protease Enzymatic Inhibition Assays

The C1s assay was a chromogenic assay modified from (34). The inhibitors that were tested were synthesized at Pfizer CP-349547, serine protease inhibitor 1, CP-013043, CP-143217, CP-669685, PHA 00737785 and C1s Protease Inhibitor or they were purchased. FUT-175 (Waterstone Technology, Indianapolis In) Adam 12 Inhibitor and 1, 10 phenanthroline (Sigma, St Louis, Mo), and Serine Protease Inhibitor 2 (Chembridge Corp, San Diego, Ca). Briefly, 800ng purified C1s (Calbiochem #204879) and the various inhibitors, dissolved in DMSO (0.5%), were incubated in 0.1 ml of 50mM Tris-HCL, 4mM CaCl2, pH 7.4 for 30 min. Chromogenic substrate (Diapharma #S-2765) 1.0 mM was added and absorbance read at 405nm. The thrombin protease inhibition was measured using the chromogenic substrate, S-2238 (Diapharma) following the manufacturer’s methods listed in the Chromogenix Substrate Booklet.

## Results

Analysis of the protease activity by IGFBP-5 zymography showed that joint fluid contained a single band (Mr 88 kDa) that cleaved IGFBP-5 (Figure 1A). Following the three step purification in which the specific activity of the proteolytic activity was increased approximately 2000 fold, repeat analysis showed the identical zymographic band and no new zymographic bands were detected (Figure 1A). This Mr estimate corresponded to the Mr estimate of C1s which cleaves IGFBP-5 (24, 28). Immunoblotting for C1s showed that both the joint fluid and the purified material contained a single immunoreactive band with an Mr estimate of 88 kDa (Figure 1B).

To determine if the pattern of proteolytic cleavage changed following purification, the molecular sizes of the IGFBP-5 cleavage fragments were estimated using the same purified chromatographic fractions. Multiple bands ranging in Mr estimates from 19–24 kDa were detected when either the non-purified joint fluid or the partially purified material was used as a source of the protease activity (Figure 2). The antibody is relatively specific for the N-terminal region of IGFBP-5 and therefore C-terminal cleavage fragments that were present in low concentrations may not be detected.

To confirm that the 88 kDa band contained C1s and determine if other proteases that were present the partially purified material was further fractionated by SDS-PAGE. Two bands Mr 90 and 86 kDa were excised, digested with trypsin and the resulting peptides were analyzed using mass spectrometry. MS/MS data was searched against the Swiss-Prot mammalian database using the search engine MASCOT. C1s was identified in each band. The only other serine protease that was detected was complement 1r which we had shown previously did not cleave IGFBP-5 (28). No peptides corresponding to tryptic fragments of the metalloproteases, or cysteine proteases were detected. Specifically HTRA-1 was not detected.

To further determine if the protease activity in unfractionated joint fluid and the purified preparation had similar properties, the effects of several protease inhibitors on IGFBP-5 proteolysis were quantified. The compounds were tested between 0.1 to 360 uM. The amount of purified enzyme that was used was determined by quantifying the amount that was required to degrade 90% of the IGFBP-5 in 4 hours. This was compared to the IGFBP-5 proteolytic activity of commercially available preparation of C1s (Table I). The results showed that for non purified joint fluid 3 of the 11 inhibitors that were tested were highly active with IC50's ranging between 0.5 and 6 uM (Table I) (Figure 3). All three compounds are serine protease inhibitors. FUT175, is a potent C1s inhibitor and CP 143217 is highly active against thrombin. CP-349547 and C1s protease inhibitor had intermediate activity. Serine Protease Inhibitor 1 had a biphasic response being highly active at 2 uM then lost activity until 100 uM was added. This is consistent with concentration dependent aggregation and subsequent loss of activity. Serine Protease Inhibitor 2 was active but aggregated at high concentrations (Table I). An MMP 2, 9 and 13 inhibitor showed no inhibition at 350 uM and an Adam4 inhibitor as well as other MMP inhibitors showed no inhibition at this concentration. An Adam 12 inhibitor had some activity at 360 uM but did not achieve 50% inhibition. Therefore FUT175, CP143217 and Serine Protease Inhibitor 1 were the most active for inhibiting the IGFBP-5 protease activity in crude joint fluid. When these compounds were tested using the purified joint fluid preparation similar results were obtained with the exception of Serine Protease Inhibitor 1 (Figure 4). CP349547, FUT175, and CP143217 were the most active and their IC50's were between 1 and 6 uM. Therefore FUT 175 and CP143217 had values that were similar to these obtained using crude joint fluid (Table I). Compound CP349547 had lower IC50 (4.0 uM) compared to its IC50 using crude synovial fluid. These results estimated from scanning densitometry are consistent with

inhibition observed in a biochemical assay using purified commercially available serum C1s (Table I). The three compounds with the greatest inhibitory activity for the purified protease all had IC50 values of 1 uM or less. These results further support the conclusion that the protease activity in partially purified material is the same as that in unfractionated joint fluid. HTRA1, was clearly detectible in the crude joint fluid by immunoblotting (Figure 5). To confirm that it had been removed during the purification process the chromatographic fractions (analyzed for C1s in Figure 1) were immunoblotted for HTRA-1. A single band (Mr estimate of 28 kDa) was detected. This corresponds to the Mr estimate of the active fragment of HTRA1 that contains its protease activity (29). This band was removed during purification and a band corresponding to this molecular weight was not detected by zymography. Moreover, based on LC-MS/MS analysis there was no detection of HTRA-1 in either of the two bands with zymographic activity ($M_r$ 88–92 kDa).

## Discussion

Inhibition of C1s activity in dog joint fluid results in increases in intact IGFBP-5 and IGF-I (24). Inhibiting cleavage of IGFBP-5 also resulted in a protective effect for articular cartilage in a dog model of osteoarthritis (24). However there have been discrepant findings regarding which serine proteases are present and active in human joint fluid. Furthermore several proteases including HTRA1 (29) Adam 9 (35), Adam 12S (36), PAPP2A (37) MMP2 and 9 (38) have been shown to cleave IGFBP-5. Our findings demonstrate that C1s is likely to account for most of the IGFBP-5 protease activity in human osteoarthritic fluid. Several findings support this conclusion. Mass spectrometric identification of the proteins from the SDS gel band that corresponded in molecular weight to the IGFBP-5 zymographic activity showed that it contained C1s. Low concentrations (e.g. 1–6 uM) of serine protease inhibitors with a high specificity towards pure C1s inhibited cleavage of IGFBP-5 by crude joint fluid. Finally the cleavage fragments generated by the purified material that was enriched in C1s and those generated by exposure to joint fluid appeared to be of similar molecular size. Taken together with our earlier published findings that C1s inhibition resulted in improvement in joint architecture in a dog model of osteoarthritis, the findings suggest that inhibition of IGFBP-5 proteolysis by inhibiting C1s may be an approach to improving articular cartilage protection during the development of OA.

IGF-I stimulates proteoglycan synthesis, chondrocyte cell proliferation and protein synthesis (1,2,4,9,10,23). Studies in animal models have shown that direct injection of IGF-I into the joint or administration of GH which results in an increase in the concentration of IGF-I in joint fluid are trophic for growth plate cartilage during normal development and for articular cartilage during the development of osteoarthritis (13,39–42). IGF-I synergizes with other growth factors such as EGF, TGFα and FGF to enhanced proteoglycan synthesis and cartilage repair. Gene therapy using adenovirus-associated IGF-I transfection experiments has shown that overexpression of IGF-I in experimental models of osteoarthritis is protective for articular chondrocytes (13–15). Strategies to decrease cytokine levels and inflammatory cell activation in combination with IGF-I therapy produce additive improvement in cartilage matrix accumulation (43). Osteogenic protein-I (OP-1) enhances the effects of IGF-I on articular cartilage and IGF-I synthesis is upregulated during articular chondrocyte repair in several animal models of arthritis (44). These findings suggest that restoration of IGF-I to normal levels or attainment of supraphysiologic concentrations in the periarticular space during repair may lead to improved outcomes.

The role of IGF binding proteins in modulating IGF-I action in cartilage has yielded conflicting results. Fernaho et al demonstrated that in a dog model of osteoarthritis IGFBP-3 and 4 were upregulated and these changes were accompanied by increases in IGF-I (45). Iwanaga et al using immunohistochemistry and in situ hybridization showed increased

expression of IGFBP-3, 4 and 5 in osteoarthritic articular cartilage as compared to control (20). Large increases in the concentrations of IGFBPs in extracellular fluid can limit IGF-I access to receptors. DeCeunick et al showed that inhibition of IGF-I association with IGFBP-3 resulted in improvement in proteoglycan synthesis in human osteoarthritic cartilage suggesting that IGFBP-3 was inhibiting IGF-I actions (22). However, studies by Duan et al suggested that IGFBP-3 had an important positive modulatory role of IGF-I activity in chondrogenesis during development (46). Kiepe et al reported that IGFBP-1, 2, 4 and 6 act exclusively as growth inhibitors of cartilage cells in vitro however they obtained more complex results with IGFPB-3 showing that if it was preincubated with growth plate chondrocytes and then IGF-I was added it could potentiate the effects of IGF-I whereas if a molar excess of IGFBP-3 was added together with IGF-I it inhibited IGF-I action (47). In contrast to these other binding proteins, these investigators determined that IGFBP-5 enhanced IGF-I stimulated growth plate chondrocyte proliferation. These investigators determined that IGF-I induced IGFBP-5 expression in cultured chondrocytes and that the increase led to enhanced chondrocyte differentiation. They also showed that IGFBP-5 potentiated the ability of IGF-I to enhance AKT activation (21). Overexpression of IGFBP-5 alone without IGF-I did not enhance chondrocyte proliferation or differentiation.

The most plausible mechanism accounting for these effects is that when IGFBP-5 binds to extracellular matrix components its affinity is lowered greater than 10 fold for IGF-I and this results in more favorable equilibrium between the IGF-I that is bound to IGFBP-5 and the IGF-I receptor (48). The concomitant association of IGF-I with IGFBP-5 protects IGF-I from being cleared rapidly from the joint space thus prolonging its ability to enhance cartilage repair. Therefore maintenance of a relatively high concentration of low affinity, intact IGFBP-5 provides a reservoir for IGF-I and this may be important for an anabolic chondrocyte response.

Identification of the protease that is degrading IGFBP-5 in cartilage has been difficult due to the multiplicity of proteases that are present during development of osteoarthritis. Metalloproteases, serine proteases and cysteine proteases have all been shown to be activated. Multiple types of proteases including proteases from all three major classes have been shown to degrade IGFBP-5, but studies with protease inhibitors using a variety of physiologic fluids related to osteoarthritis, i.e. culture supernatants, osteoarthritic joint fluid or cartilage extracts and synovial cell culture fluid, have shown that inhibition of serine proteases results in increases in IGF-I concentrations. The serine proteases that have been implicated in osteoarthritis have focused on the urokinase-plasminogen system. Although components of that system can degrade IGFBP-5, studies to identify IGFBP-5 proteases in joint fluid have not confirmed they account that account for the IGFBP-5 proteolytic activity.

A serine protease that deserves special consideration is HTRA1. Purified HTRA1 degrades IGFBP-5 (29) and HTRA1 is present in human osteoarthritic joint fluid. Two studies have shown that HTRA1 is upregulated following articular cartilage damage in experimental animal models of arthritis and in human OA joint fluid (30,31). Grau et al demonstrated that HTRA1 was increased 7 fold in synovial fluid from rheumatoid and osteoarthritic patients (31). We could not confirm that HTRA1 accounted for the IGFBP-5 protease activity in joint fluid. The electrophoretic mobility of HTRA1 was very different from the band accounted for by zymographic activity. Secondly immunoblotting of the joint fluid material at each purification step for HTRA revealed that most of the immunoreactivity was removed during the final purification step, whereas the IGFBP-5 protease activity was retained. Third, mass spectrometry sequence analysis showed that the gel band that contained the proteolytic activity that accounted for IGFBP-5 proteoase activity did not contain HTRA-1. Finally specific protease inhibitors that had poor activity against purified HTRA1 (data not shown)

were potent inhibitors of the IGFBP-5 protease activity in crude joint fluid. These findings suggest that although HTRA1 is present in joint fluid and is proteolytically active, it is not primarily responsible for degrading IGFBP-5. This could be due to affinity differences, that is the affinity of C1s for IGFBP-5 may be substantially higher than HTRA1 or to the presence of endogenous inhibitors in joint fluid that preferentially alter the activity of HTRA1 as opposed to C1s. In support of differences in affinity, C1s concentrations as low as 3 nM degrade IGFBP-5 (28) whereas 100 nM HTRA1 was required to detect significant activity (29).

In summary, our findings support the conclusion that C1s accounts for the proteolytic activity in human osteoarthritic fluid for IGFBP-5. Because increasing the amount of intact IGFBP-5 increases the total IGF-I concentration and enhances IGF-I actions in cell types that are present in the joint space, it appears to be chondroprotective in experimental animal models of osteoarthritis. This suggests that specific strategies to inhibit C1s activity may positively modulate the reparative response in human OA.

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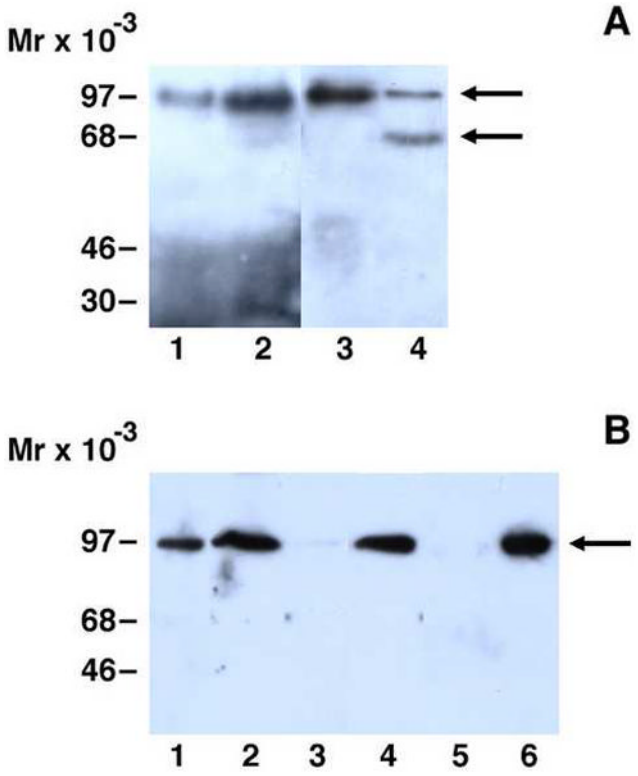



**Figure 1.**

A. Zymographic analysis of IGFBP-5 protease activity in C1s in crude and purified joint fluid. Human joint fluid was purified as described in methods. The figure shows the IGFBP-5 protease activity as assessed by IGFBP-5 zymography in crude synovial fluid (lane 1) and highly purified fluid (lane 2). A separate sample of the highly purified preparation is shown in lane 3. A control sample of conditioned medium from a tumor cell line that contained both C1s and unidentified IGFBP-5 protease other than C1s (lower arrow) is shown for comparison in lane 4. The human synovial fluid samples contain a single 88 kDa band as denoted by the upper arrow. No other bands were detected.

B. Assessment of C1s abundance by immunoblotting. The same joint fluid that was analyzed in Figure 1A was also analyzed by immunoblotting for C1s. The results show that a single band is detectible in crude joint fluid (denoted by arrow) that has an Mr estimate corresponding to the 88 kDa band noted on zymography (panel A, lane 1). An aliquot of the active material that was concentrated during each chromatographic step was analyzed in lanes 2–6. Lane 2, butyl sepharose eluate, lane 3, wheat germ agglutinin (WGA) flow through, lane 4 WGA eluate, lane 5, concanavalin-A (Con-A) flow through, lane 6, Con-A eluate.

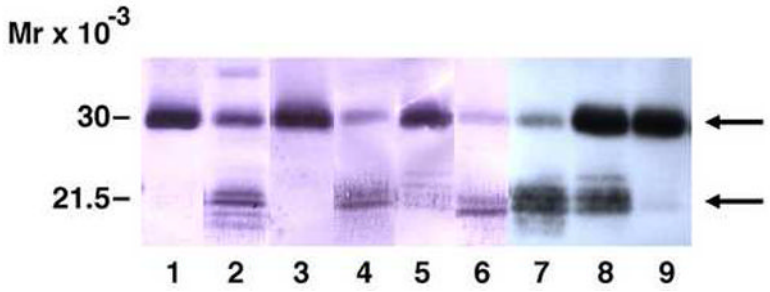



**Figure 2.**

IGFBP-5 cleavage. The crude joint fluid and the purified material obtained after each purification step were incubated with a known concentration of intact IGFBP-5 as described in methods. The products of the reaction were analyzed by immunoblotting for IGFBP-5. Lane 1, human IGFBP-5 standard, 5 ng. Lane 2, nonpurified human synovial fluid. The arrows denote the position of intact IGFBP-5 (upper) and the position of the IGFBP-5 fragments (lower). The chromatographic fractions are shown in lanes 3–9. Lane butyl sepharose flow through, lane 4, butyl sepharose eluate, lane 5, WGA flow through, lane 6, WGA eluate. Lane 7 Con-A eluate 5 ul, lane 8 Con-A eluate 2 ul, lane 9 Con-A flow through.

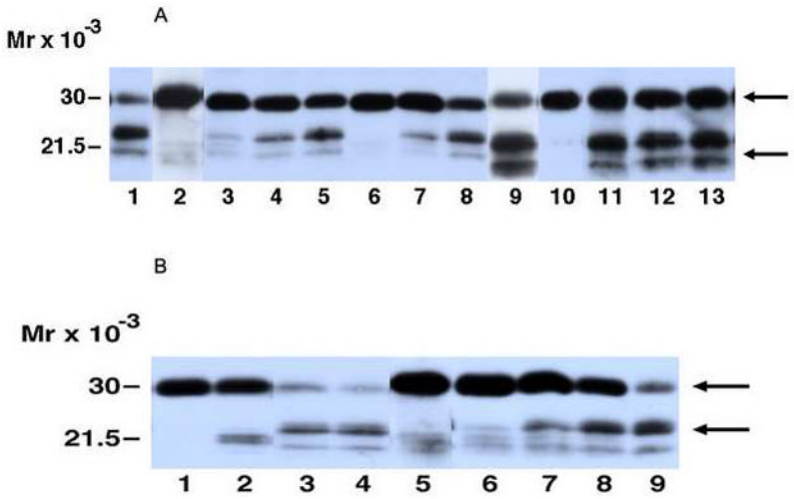



**Figure 3.**

A. The effect of protease inhibitors on IGFBP-5 cleavage by proteases in human synovial fluid. Only those inhibitors that were active using reasonably low concentrations are shown. The effect of nonpurified synovial fluid is shown in lane 1. Lanes 2–12 contained the same amount of synovial fluid. Compound CP143217 is shown in lanes 2–5, lane 2, 45 uM, lane 3, 24 uM, lane 4, 6 uM, lane 5, 2 uM. Lanes 6–8, FUT175, lane 6, 24 uM, lane 7, 6 uM, lane 8, 2 uM. Lane 9, control HSF, lanes 10–13, CP349547, lane 10, 360 uM, lane 11, 45 uM, lane 12, 6 uM, lane 13, 2 uM. The arrows indicate the position of intact IGFBP-5 (upper) and the cleavage products (lower). B. Effect of inhibitors on IGFBP-5 proteolysis by human synovial fluid. Additional inhibitors were tested as in figure 3. Lanes 1–3, C1s Protease Inhibitor, lane 1, 350 uM, lane 2, 70 uM, lane 3, 5 uM lane 4, control HSF, Lanes 5–8, Serine Protease Inhibitor 1, lane 5, 45 uM, lane 6, 2 uM, lane 7, 0.5 uM, lane 8, 0.1 uM, lane 9, control HSF.

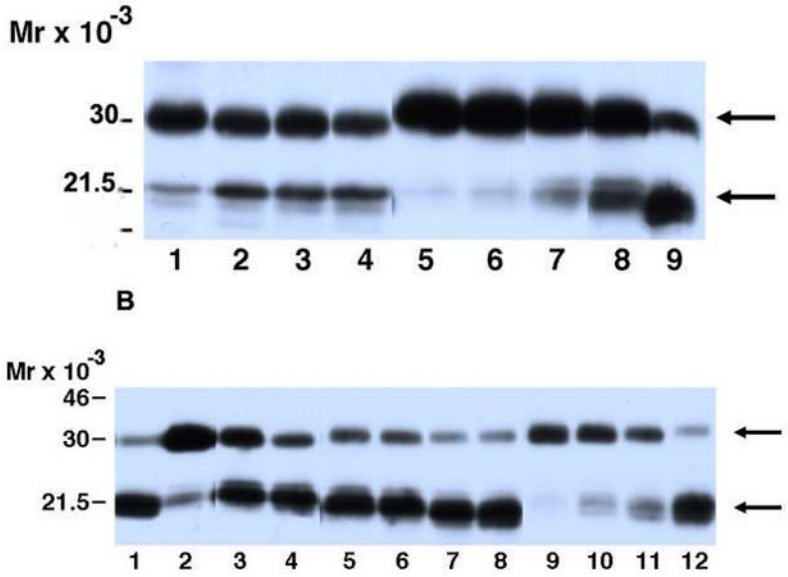



**Figure 4.**

A. Effect of inhibitors on cleavage of IGFBP-5 by the purified enzyme. The enzyme preparation that had been purified through the conconvalin A step was used as a source of protease and the effect of the same inhibitors that were analyzed in Figure 3 was assessed. Lanes 1–9 purified protease, Lanes 1–3, C1s Protease Inhibitor, lane 1, 100 uM, lane 2, 25 uM, lane 3, 5 uM, lane 4 control, purified protease. Lanes 5–8 FUT175, lane 5, 100 uM, lane 6, 10 uM, lane 7, 2.5 uM, lane 8, 0.62 uM, lane 9 control protease. B. Effect of protease inhibitors on the activity of the purified enzyme. As in Figure 5 the enzymatic activity that had been purified through the Con A step was incubated with the protease inhibitors and IGFBP-5 as described in methods. Lane 1–12 purified protease, lanes 2–4, CP143217, lane 2, 100 uM, lane 3, 6 uM, lane 4, 2 uM, lanes 5–8 Serine Protease Inhibitor 1, lane 5, 100 uM, lane 6, 45 uM, lane 7, 0.5 uM, lane 8, control protein, lanes 9–11 CP349547, lane 9, 24 uM, lane 10, 6 uM, lane 11, 2 uM, lane 12, control purified protease. The arrows show the position of intact IGFBP-5 (upper) and the 22 kDa fragment (lower).

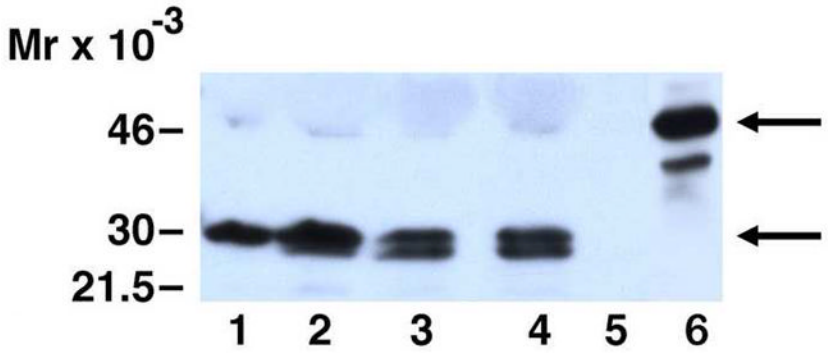
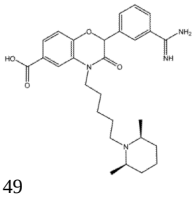
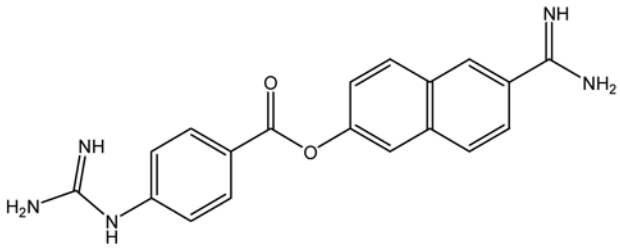
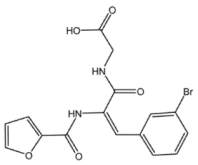
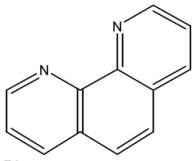
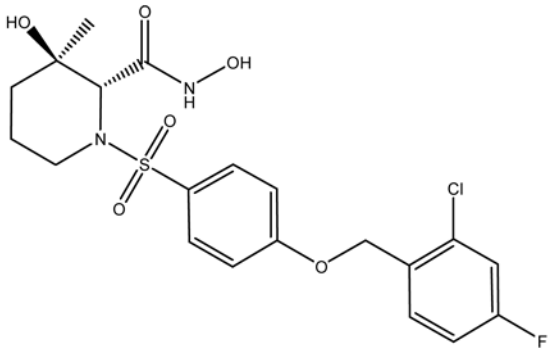
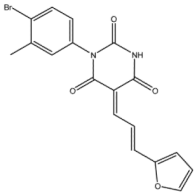
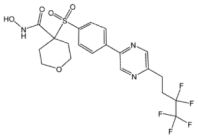
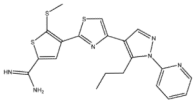



**Figure 5.**

Abundance of HTRA-1 during purification. The fractions that were analyzed for IGFBP-5 protease activity at each purification step were analyzed for the abundance of HTRA-1 by immunoblotting as described in methods. Lane 1, nonpurified joint fluid, lane 2, WGA column fall through, lane 3, WGA eluate, lane 4, Con-A, fall through, lane 5, Con-A eluate. The arrows denote the position of the active fragment of HTRA-1 that contains the protease activity. Lane 6 shows an intact HTRA-1 standard for comparison. The same amount of total protein that was immunoblotted for C1s was also immunoblotted for HTRA-1. The arrow denotes the position of the major cleavage fragment of HTRA-1 that contains the catalytic activity of the enzyme (31). The results show that most of the HTRA-1 activity is eliminated in the flow through fractions from the wheat germ, agluttin and the Con-A columns. No HTRA-1 immunoreactive fraction corresponds to the position of the zymographic activity noted in Figure 1.

## TABLE I

| Compound Information/Structure | Thrombin $IC_{50}$ (μM) | Human Joint Fluid $IC_{50}$ (μM) | Purified Protease $IC_{50}$ (μM) | Purified C1s $IC_{50}$ (μM) |
|---|---|---|---|---|
| CP-349547 Serine protease inhibitor | 0.121 | 20 | 4 | 1 |
|  49 Serine protease inhibitor 1* | | 0.5 | 100 | >100 |
| CP-013043 Serine Protease Inhibitor | 0.140 | >360 | 100 | >100 |
| CP-143217 Serine Protease Inhibitor | 0.281 | 6 | 6 | 1 |
|  50 FUT175 Serine Protease Inhibitor | 3400 | 3 | 1 | 0.070 |
|  51 Adam 12 Inhibitor | Not tested | >360 | No inhibition | >100 |
|  52 1,10-phenanthroline, MMP Inhibitor | Not tested | No Inhibition | No inhibition | Not tested |

| Compound Information/Structure | Thrombin $IC_{50}$ (μM) | Human Joint Fluid $IC_{50}$ (μM) | Purified Protease $IC_{50}$ (μM) | Purified C1s $IC_{50}$ (μM) |
|---|---|---|---|---|
|  53 CP-669685 Adam 4 Inhibitor | Not tested | No Inhibition | No inhibition | >100 |
|  Serine Protease Inhibitor 2* | Not Tested | 90 | 80* | Not tested |
|  54 PHA-00737785, MMP 2, 9, 13 Inhibitor | Not Tested | No Inhibition | No Inhibition | >100 |
|  55 C1s Protease Inhibitor | >30 | 30 | 25 | 1.03 |

*Induced aggregation