The Role of Human HtrA1 in Arthritic Disease*

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Sandra Grau‡1, Peter J. Richards‡1, Bridgeen Kerr‡, Clare Hughes§, Bruce Caterson*, Anwen S. Williams§, Uwe Junker§, Simon A. Jones‡, Tim Clausen¶ and Michael Ehrmann‡2

From the ‡School of Biosciences, Cardiff University, Cardiff CF10 3US and the §Rheumatology Research Laboratory, Cardiff University, Cardiff CF14 4XN, United Kingdom, ¶Novartis Pharmaceuticals, CH-4002 Basel, Switzerland, and the †Institute for Molecular Pathology, A-1030 Vienna, Austria

Human HtrA1 belongs to a widely conserved family of serine proteases involved in various aspects of protein quality control and cell fate. Although HtrA1 has been implicated in the pathology of several diseases, its precise biological functions remain to be established. Through identification of potential HtrA1 targets, studies presented herein propose that within the context of arthritis pathology HtrA1 contributes to cartilage degradation. Elevated synovial HtrA1 levels were detected in fluids obtained from rheumatoid and osteoarthritis patients, with synovial fibroblasts identified as a major source of secreted HtrA1. Mass spectrometry analysis of potential HtrA1 substrates within synovial fluids identified fibronectin as a candidate target, and treatment of fibroblasts with recombinant HtrA1 led to the generation of fibronectin-degradation products that may be involved in cartilage catabolism. Consistently, treatment of synovial fibroblasts with HtrA1 or HtrA1-generated fibronectin fragments resulted in the specific induction of matrix metalloprotease 1 and matrix metalloprotease 3 expression, suggesting that HtrA1 contributes to the destruction of extracellular matrix through both direct and indirect mechanisms.

Human HtrA1 (L56) is a member of the HtrA (High temperature requirement) family of serine proteases, a well defined group of proteases sharing many of the characteristics associated with bacterial HtrAs (1). Such features include a highly conserved trypsin-like serine protease domain and at least one PDZ domain at the C terminus. In addition, HtrA1 contains an insulin-like growth factor-binding protein domain and a Kazal-type serine protease inhibitor motif at its N terminus (2). Originally identified as a gene down-regulated in SV40-transformed fibroblasts (2), HtrA1 has since been implicated in the modulation of various disease pathologies. Recent reports suggest that HtrA1 plays a protective role in various malignancies because of its tumor-suppressive properties (3–6). Studies have shown that HtrA1 is down-regulated in cancerous tissue as compared with normal tissue and that overexpression results in the inhibition of tumor cell growth and proliferation both in vitro and in vivo (5). In contrast to tumor tissue, HtrA1 expression is up-regulated in skeletal muscle of Duchenne muscular dystrophy (7) and in cartilage of osteoarthritic joints (8). Therefore, up-regulation of HtrA1 in osteoarthritic joints may contribute to the development of this debilitating disease.

Progressive degradation of components of the extracellular matrix plays an important role in the pathogenesis of arthritic diseases (9, 10). In osteoarthritis or rheumatoid arthritis, fibroblast and chondrocyte (9, 18, 19). Within the cartilage matrix, interstitial collagens are the main targets of degradative collagenases such as MMP-1 (collagenase-1) (11, 12). The primary function of these MMPs is in the degradation of native fibillary collagen, resulting in the generation of collagen fragments that are then further cleaved by gelatinases, MMP-2 and MMP-9, and stromelysin (MMP-3) (12). However, for collagenases to gain access to these substrates, small proteoglycans and interstitial collagens must first be removed (12). Recently, it was suggested that several proteoglycans and glycoproteins in the extracellular matrix may serve as potential substrates for HtrA1 (20–22) and that this protease may therefore be pivotal in the onset of destructive joint pathology seen in arthritic disease. In the present study, we have demonstrated a potential direct and indirect involvement of HtrA1 in cartilage destruction in arthritic diseases.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified eagle medium, Dulbecco’s calcium- and magnesium-free phosphate-buffered saline, heat-inactivated bovine calf serum, l-glutamine, penicillin and streptomycin, collagenase Type I were obtained from Invitrogen. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, thiazolyl blue, 3,3′,5,5′-tetramethylbenzidine, hydrocortisone, insulin, and transferrin were obtained from Sigma. Human recombinant interleukin 1β was obtained from R&D Systems Inc. Horseradish peroxidase-conjugated streptavidin was from Amersham Biosciences, and fibronectin was purchased from Chemicon. All oligo-primers were purchased from MWG Biotech.

Antibodies—A monoclonal HtrA1 antibody was generated against recombinant purified HtrA1 (amino acids 141–480) using previously described approaches (23). Polyclonal HtrA1 antibody was produced by injecting purified recombinant HtrA1 (amino acids 141–480) from Escherichia coli into rabbits.

Isolation of Human Synovial Fibroblasts (HSF)—HSF were isolated, harvested, and cultured using a method previously described (24). Briefly, synovial tissue was obtained after synovectomy from patients with osteoarthritis or rheumatoid arthritis under approval of the local Ethics Committees. Samples were washed with Dulbecco’s calcium- and magnesium-free phosphate-buffered saline prior to digestion with collagenase (750 units/ml in phosphate-buffered saline) for 1 h at 37 °C. After digestion, the synovial fibroblasts were expanded in culture flasks.
containing Dulbecco’s modified Eagle’s medium and nutrient mix F12 (1:1) supplemented with 10% fetal calf serum, penicillin (50 international units/ml), streptomycin (50 μg/ml), l-glutamine (0.3 mg/ml), hydrocortisone (4 μg/ml), insulin (250 μg/ml), and transferrin (250 μg/ml). Cells were grown in a humidified incubator at 37 °C containing 5% CO2 in air. At least four separate cell lines were cultured and used between passages 3 and 5.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Cell Proliferation Assay—Cell viability was assessed using the 3-(4,5-di- methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (25). This assay was used throughout cell culture experiments as standard to confirm that cell numbers/viability were comparable.

HtrA1 ELISA—HtrA1 protein levels within synovial fluid and cultured supernatant samples were determined using an HtrA1-specific ELISA developed in-house. Briefly, ELISA plates were coated overnight with monoclonal α-HtrA1 (1:100) and blocked with 5% bovine serum albumin/phosphate-buffered saline. Plates were washed with 0.05% Tween/phosphate-buffered saline and incubated with samples for 2 h at 30 °C. After washing, polyclonal α-HtrA1 (1:500) was added for 1 h at 30 °C followed by a biotin-conjugated swine α-rabbit (1:5000) (Dako Cytomation) for 1 h at 30 °C. HtrA1 was detected using horseradish peroxidase-conjugated streptavidin (1:5000). Plates were developed using 3’,5’-5,5’-tetramethylbenzidine in 100 mM citric acid, 0.1% H2O2, pH 3.95. The reaction was stopped with 7% H2SO4 and optical densities determined at 450 nm using a plate reader (Dynex). Purified recombinant HtrA1 (amino acids 141–480) was tested at concentrations ranging from 156 pg/ml to 10 ng/ml to generate a standard curve.

Cloning, Expression, and Purification of HtrA1—The expression vector pCTH1 is a derivative of pQE60 (Qiagen) containing the lacIα gene (26). pSG7 expresses codons 141–480 of HtrA1 under tac promoter control (isopropyl β-D-galactosidase inducible). It was constructed by cloning a PCR fragment via NcoI/BglII into pCTH1. E. coli strain CLC198 (MC4100 incB) was lysed by French pressing in buffer I containing casein as substrate (Bio-Rad) and developed following the manufacturer’s instructions (Invitrogen). cDNA was synthesized from pd(N)-primed mRNA reverse transcribed using M-MLV superscript reverse transcriptase as previously described (27). Reaction mixtures for PCR consisted of 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100 and 2.5 mM MgCl2, 2 mM deoxynucleotides, 1 pmol of each primer, 16 pg of cDNA, and 0.5 units of Tag polymerase (Promega). PCR was performed on a thermal cycler (Tersonal; Biometra). Human mRNA specific for the housekeeping gene α-actin was measured and used as an internal control. Reaction times were optimized for α-actin, MMP-1, -2, -3 and TIMP-1, -2, and -3 and were 94 °C for an initial 2 min, followed by either 30 cycles for α-actin, MMP-2, TIMP-1, and TIMP-2 or 40 cycles for MMP-1 and -3 of 15 s, 53 °C for 30 s, and 72 °C for 1 min. The reaction was completed with a 7-min extension at 72 °C. The primers for α-actin (204 bp) were 5’-GGA GCA ATG ATC TTG ATC TT-3’ for the upstream primer and 5’-TCC TGA GGT ACG GTT CCT TTC-3’ for the downstream primer; MMP-1 (449 bp) 5’-TCC CAG CGA CTC TAG AAA CAC AAG-3’ for the upstream primer and 5’-CCG ATG ATC TCC CCT GAC AAA AG-3’ for the downstream primer; MMP-2 (619 bp) 5’-TTT TTT TCT CGA ATC CAT GAT GG-3’ for the upstream primer and 5’-CTG GTG CAG CTC TCA TAT TT-3’ for the downstream primer; MMP-3 (434 bp) 5’-AGT TTC AGT GTT GGC TGA G-3’ for the upstream primer and 5’-CAG GTG TGG AGT TTC TCC GAG-3’ for the downstream primer; TIMP-1 (320 bp) 5’-CAG GAA TTC TCC GAG CCG GAT CG-3’ for the upstream primer and 5’-GGT TAC ACG CAT GAC GAA GCC-3’ for the downstream primer; TIMP-2 (410 bp) 5’-CTT CAT CCT CAT CTC CTG CAT CG-3’ for the upstream primer and 5’-CAG GGC TAG TGT TTG GAC TG-3’ for the downstream primer. Ethidium bromide (0.005%)-stained agarose gels were photographed using the GelDoc-It imaging system (Lecon-Luc-PLC).

Quantification of Secreted MMP-3—MMP-3 protein levels in culture supernatants were determined using an MMP-3-specific ELISA kit according to the manufacturer’s instructions (R&D Systems Inc.).

Effect of Fibronectin Fragments on MMP and TIMP Expression—To prepare fibronectin fragments, 10 μg of fibronectin was incubated with 5 μg of HtrA1 for 16 h at 37 °C in buffer in 50 mM Tris-HCl, pH 8.5, 150 mM NaCl. Subsequently, these samples were applied with and without 5 μM HtrA1 inhibitor to synovial fibroblasts for 24 h before determining MMP and TIMP mRNA levels by RT-PCR.

Statistical Analysis—Two-tailed Student’s t-test was used to determine statistical significance between values. A p value of <0.05 was considered statistically significant. Values are expressed as the mean ± S.E.

RESULTS
Hu et al. (8) reported a 7-fold increase in both the expression of HtrA1 mRNA and HtrA1 protein in cartilage explants from osteoar-
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FIGURE 1. Detection of HtrA1 in human synovial fluid. HtrA1 levels in synovial fluid from non-arthritic (n = 6), OA (n = 22), and RA (n = 38) knee joints were determined using the HtrA1-specific ELISA (± S.E. triplicates), p < 0.05 determined by Student’s t-test. Standard curve of the HtrA1-specific ELISA is shown in the inset. R², square of correlation coefficient.

FIGURE 2. Detection of HtrA1 in supernatants of cultured human foreskin fibroblasts (HFF) and OA and RA human synovial fibroblasts. HFF and HSF of OA (n = 4) and RA (n = 4) knee joints were cultured under serum-free conditions for 24, 48, and 72 h. HtrA1 levels in supernatants were determined by HtrA1-specific ELISA. *, p < 0.05, **, p < 0.01 as determined by Student’s t-test.

arthritic patients as compared with normal individuals. To investigate the potential importance of HtrA1 in the progression of arthritis, initial studies were carried out to determine the level of HtrA1 in synovial fluids. In the present study, the role of HtrA1 in arthritic diseases was further investigated and its possible mechanisms of action elucidated.

Identification of HtrA1 in Synovial Fluid—HtrA1 levels within synovial fluids from either OA or RA patients were determined by ELISA using purified recombinant HtrA1 as a standard reference (Fig. 1, inset). HtrA1 levels in arthritic patients were compared with those detected in synovial fluids taken from non-arthritic trauma patients. HtrA1 levels were elevated in OA (38 ± 6 ng/ml) and RA (19 ± 4 ng/ml) synovial fluids as compared with non-arthritic individuals (5 ± 1 ng/ml) (Fig. 1).

HtrA1 Secretion by Human Synovial Fibroblasts—To identify potential sources of HtrA1 in synovial fluids, the secretion of HtrA1 by HSF isolated from either OA or RA patients was determined using the HtrA1-specific ELISA. Levels of HtrA1 secreted by human foreskin fibroblasts were also analyzed and served as a non-arthritic control. HtrA1 levels were significantly elevated in supernatants from OA and RA HSF as compared with human foreskin fibroblasts at all time points tested (Fig. 2).

Production, Purification, and Inhibition of Recombinant HtrA1—To further analyze the effects of HtrA1 in the context of arthritis, we generated a recombinant His-tagged HtrA1 lacking the N-terminal insulin-like growth factor-binding protein and serine protease inhibitor domain in E. coli (Fig. 3A). Affinity-purified HtrA1 was >98% pure as determined by SDS-PAGE (Fig. 3B, lane 1) and was recognized by the monoclonal HtrA1 antibody (lane 2). In addition, this HtrA1 construct was confirmed to be proteolytically active as shown by zymography (lane 3). This truncated version of HtrA1 is thought to be of physiological relevance as HtrA1 possesses autoproteolytic activity generating N-terminal truncations in in vitro translation (8) as well as cell culture systems (data not shown). An additional tool for these studies was a potent inhibitor of HtrA1 that was obtained from a high throughput screen (Fig. 3C). In the presence of this HtrA1 inhibitor, proteolytic activity was inhibited in a dose-dependent manner with an IC₅₀ of 0.21 μM as determined by HtrA1-dependent digestion of resorufin-labeled casein (Fig. 3D).

Identification of Potential Substrates of HtrA1—The identification of substrates naturally occurring within the joint would be beneficial for investigating the role of HtrA1 in this destructive disease. The potential of cartilage matrix degradation has been previously demonstrated by the ability of HtrA1 to digest small proteoglycans such as decorin and biglycan (20, 22). To identify possible substrates of HtrA1 within arthritic joints, OA and RA synovial fluids low in HtrA1 (OA, 6.2 ng/ml; RA 2.2 ng/ml) were digested with purified recombinant HtrA1, and degraded proteins were identified by SDS-PAGE followed by mass spectrometry. Among the candidate substrates identified, fibronectin was considered to be of particular interest because of its involvement in maintenance of cartilage matrix integrity through its interaction with collagen (28). In addition, elevated levels of fibronectin fragments produced following proteolytic degradation have been detected in both OA and RA synovial fluids (29–34). These fragments may play an important role in arthritic diseases because of their ability to stimulate chondrocytes and synovial fibroblasts to produce MMPs (35–37). Protease assays with purified components were performed to confirm fibronectin degradation. Various amounts of recombinant HtrA1 were incubated with 10 μg of fibronectin for 3 and 18 h at 37 °C. A fragment of fibronectin migrating at ~30 kDa could be detected after 3 h of incubation with 3 and 5 μg of HtrA1 (Fig. 4A). Further incubation of fibronectin with HtrA1 for 18 h led to generation of additional fibronectin fragments ranging from 50 to 175 kDa that increased in intensity with increasing amounts of HtrA1 (Fig. 4A). The most prominent fibronectin fragment generated after 18 h was the 30-kDa fragment. Fibronectin degradation was completely abolished by addition of 5 μM HtrA1 inhibitor. In addition, inhibition of HtrA1-dependent fibronectin digestion was investigated by preincubating HtrA1 with 1, 3, or 5 μM HtrA1 inhibitor prior to adding fibronectin. Degradation of fibronectin was completely abolished by addition of 3 and 5 μM HtrA1 inhibitor (Fig. 4B). The appearance of HtrA1 as one or two bands after prolonged incubation is due to its autoproteolytic activity. Inhibition of HtrA1 also inhibits autoproteolytic activity resulting in only one HtrA1 band.

Effect of Recombinant HtrA1 on MMP/TIMP Production by HSF—Fibronectin fragments are present in micromolar levels in synovial fluid of arthritic joints and have been shown to up-regulate MMP production in human synovial fibroblasts and chondrocytes (34, 35, 37, 38). To investigate the potential regulatory effects of HtrA1 on cellular functions...
resulting from the production of fibronectin fragments, we examined
MMP expression in HSF following incubation with recombinant
HtrA1. In addition, we investigated the effects of HtrA1 on expression of
the naturally occurring inhibitors of MMPs (tissue inhibitor of matrix
metalloproteinases, TIMPs). Both OA and RA HSF were incubated for
24 h in serum-free conditions with and without increasing amounts of
recombinant HtrA1. The expression of MMP-1, -2, -3 and TIMP-1 and
-3 was determined by semi-quantitative PCR. MMP-1 and -3 mRNA
levels were markedly increased in both OA and RA HSF treated with
HtrA1. In contrast, expression of MMP-2, TIMP-1, and TIMP-3

FIGURE 3. Production of HtrA1 and inhibition of its proteolytic activity. A, schematic representa-
tion of full-length HtrA1 (upper panel) and truncated version (amino acids 141–480) containing
the proteolytic domain and the PDZ domain (lower panel). ss, signal sequence; IGFBP, insulin-like
growth factor-binding protein domain; KI, Kazal-type inhibitor domain. B, purified recombinant
HtrA1 (2 μg) was loaded on a 10% SDS-gel and stained with Coomassie Blue (lane 1) and analyzed
by Western blot using the monoclonal HtrA1 anti-
body (lane 2). HtrA1 (2 μg) proteolytic activity was
determined by zymography (lane 3). White areas
indicate degradation of casein. C, structural model
of the HtrA1 inhibitor. D, HtrA1 activity was deter-
mined by preincubation of purified HtrA1 (3 μg) with various concentrations of HtrA1 inhibitor in
50 mM Tris-HCl, pH 8.5, 150 mM NaCl using resoru-
fine-labeled casein as a substrate, and the IC50 was
calculated.

FIGURE 4. Fibronectin degradation. A, purified
fibronectin (10 μg) and purified recombinant
HtrA1 were incubated at 37 °C in 50 mM Tris-HCl,
pH 8.5, 150 mM NaCl. Samples were loaded on a
Schaegger gel and stained with Coomassie Blue.
When indicated, HtrA1 was preincubated with
HtrA1 inhibitor (5 μM) for 20 min prior to adding
fibronectin. B, purified fibronectin (10 μg) and
HtrA1 (5 μg) were incubated with or without
HtrA1 inhibitor under the conditions described for
panel A.
remained unaffected by HtrA1 (Fig. 5, A and B). These stimulatory effects of HtrA1 were almost completely abolished following addition of 5 μM HtrA1 inhibitor (Fig. 5, A and B), suggesting that the proteolytic activity of HtrA1 is crucial for up-regulation of MMPs. In contrast, the HtrA1 inhibitor had no effect on the regulation of MMP synthesis in response to the pro-inflammatory cytokine interleukin 1 (Fig. 5C), confirming the specificity of the HtrA1 inhibitor. To determine whether the stimulatory effects of HtrA1 on MMP regulation could also have been observed at the protein level, MMP-3 release was monitored by a specific ELISA. MMP-3 was constitutively secreted by both OA (130 pg/ml) and RA (70 pg/ml) HSF. Untreated (control) cells were compared with cells treated with HtrA1 or HtrA1 plus HtrA1 inhibitor. **, p < 0.005; ***, p < 0.0001 as determined by Student’s t-test. Concentrations used were 5 μg/ml HtrA1 and 5 μM HtrA1 inhibitor. Incubation times were 24 h.

**DISCUSSION**

HtrA1 levels in cartilage explants from OA patients have previously been shown to be increased 7-fold as compared with cartilage from non-arthritis individuals (8). In addition, in vitro studies have demonstrated that cartilage damage can induce a significant increase in HtrA1 production by resident chondrocytes (20). By using an HtrA1-specific ELISA, we have demonstrated that HtrA1 levels are also increased 3–7-fold in the synovial fluids from both OA and RA patients, with a significantly higher level of HtrA1 being detected in OA synovial fluid. Thus, HtrA1 levels could serve as an additional marker for diagnosis of disease. Further analyses revealed that HSF extracted from OA and RA joint tissue constitutively secrete HtrA1. This up-regulation of HtrA1 production appeared to be disease specific, as human foreskin fibroblasts secreted 2–3-fold less HtrA1 than OA and RA HSF. The secretion of HtrA1 by OA and RA HSF suggests that the synovial membrane may also be an important source of HtrA1 within the arthritic joint, in addition to the articular cartilage. Although both OA and RA HSF secrete...
HtrA1 is the highly active state of OA chondrocytes that accounts for the differences seen in the levels of HtrA1 in OA and RA synovial fluid (20).

Destruction of articular cartilage is a common feature of OA and RA (9, 10). We identified the extracellular matrix glycoprotein fibronectin as a natural substrate of HtrA1, suggesting a direct role of HtrA1 in matrix degradation. HtrA1 effectively degraded purified human fibronectin, generating fragments of various sizes including several prominent fragments ranging from 83 to 170 and 29–30 kDa. Elevated levels of fibronectin fragments ranging from 24 to 200 kDa have been identified both in OA and RA synovial fluid in micromolar concentrations (34, 38) and are involved in the regulation of numerous cellular activities (40, 41). The central involvement of fibronectin fragments in cartilage catabolism is highlighted by their ability to decrease proteoglycan synthesis (42) and enhance the release of several MMPs (34, 37). In the present report, we have demonstrated that HtrA1 has the potential to up-regulate MMP expression and secretion in arthritic joints through activation of HSFs. Regulation of MMP-1 and -3 expression in HSF by HtrA1 was shown to be dependent on the production of fibronectin fragments. Therefore, we suggest that HtrA1 degrades fibronectin present within the cell culture system and the resulting fibronectin fragments instigate the expression and secretion of MMPs. Additional evidence was provided from the findings that neither HtrA1 nor fibronectin fragments had any effect on TIMP-1 and -3 expression by HS as has previously been reported (34).

The present study provides evidence for a detrimental role of HtrA1 in both OA and RA, leading to a working hypothesis for its biological functions in this context. Not only does HtrA1 have the potential to directly degrade cartilage through proteolytic cleavage of extracellular matrix components such as fibronectin, cartilage oligomeric matrix protein, biglycan, decorin, fibromodulin, aggrecan, and reduced collagen (20–22), but it seems also to act indirectly through its ability to stimulate the overproduction of MMPs by synovial fibroblasts. Therefore, specific inhibition of HtrA1 production or activity in arthritic joints may serve as a novel therapeutic strategy for treatment of arthritic diseases.

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