Chondroadherin Fragmentation Mediated by the Protease HTRA1 Distinguishes Human Intervertebral Disc Degeneration from Normal Aging*

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Background: Little is known about the molecular mechanisms involved in intervertebral disc degeneration (IVD).

Results: CHAD fragmentation is found only in degenerate IVDs. HTRA1 is capable of generating the in vivo fragment.

Conclusion: CHAD fragmentation can be a marker of degeneration, distinguishing between aging and degeneration.

Significance: Inhibiting HTRA1 activity could be of value to slow down disc degeneration without influencing normal turnover.

Chondroadherin, a member of the leucine-rich repeat family, has previously been demonstrated to be fragmented in some juveniles with idiopathic scoliosis. This observation led us to investigate adults with disc degeneration. Immunoblotting analysis demonstrated that non-degenerate discs from three different age groups show no chondroadherin fragmentation. Furthermore, the chondroadherin fragments in adult degenerate disc and the juvenile scoliotic disc were compared via immunoblot analysis and appeared to have a similar size. We then investigated whether or not chondroadherin fragmentation increases with the severity of disc degeneration. Three different samples with different severities were chosen from the same disc, and chondroadherin fragmentation was found to be more abundant with increasing severity of degeneration. This observation led us to the creation of a neoepitope antibody to the cleavage site observed. We then observed that the cleavage site in adult degenerate discs and juvenile scoliotic discs was identical as confirmed by the neoepitope antibody. Consequently, investigation of the protease capable of cleaving chondroadherin at this site was necessary. In vitro digests of disc tissue demonstrated that ADAMTS-4 and -5; cathepsins K, B, and L; and MMP-3, -7, -12, and -13 were incapable of cleavage of chondroadherin at this site and that HTRA1 was indeed the only protease capable. Furthermore, increased protein levels of the processed form of HTRA1 were demonstrated in degenerate disc tissues via immunoblotting. The results suggest that chondroadherin fragmentation can be used as a biomarker to distinguish the processes of disc degeneration from normal aging.

Intervertebral disc (IVD) degeneration is present in the adult with degenerative disc disease (DDD) and at the apex of the spinal curves in adolescents with adolescent idiopathic scoliosis (AIS). It is characterized by structural failure and loss of IVD height due to proteolytic degradation of the extracellular matrix (1). Lower back pain in adult individuals is commonly associated with IVD degeneration (2). The societal and individual burdens for lower back pain are significant in Western society, putting both physical and economic stress on the patient (3).

At present, little is known about the molecular mechanisms involved in IVD degeneration and how these may differ from normal turnover of the tissue. We have previously shown that chondroadherin (CHAD) is fragmented in degenerate IVD tissue from patients with AIS but remains intact in macroscopically normal discs from such patients (4). CHAD may serve as a marker of degeneration.

CHAD is a leucine-rich repeat protein mainly expressed in cartilaginous tissues where it is located close to cells (4). It has the ability to bind triple helical collagen (5) and interact with cells via α2β1 integrin (6) as well as via cell surface heparan sulfate proteoglycans (7). Interactions of CHAD with cells have been shown to lead to a variety of cellular responses with activation of intracellular signaling and changes in the cytoskeleton depending on which of the receptors alone or in combination are involved (7, 8). CHAD clearly has the potential to influence cell metabolism and affect matrix homeostasis. CHAD is also expressed in other tissues that experience load, such as bone and tendon, albeit in a lower abundance (9–11). Thus, CHAD shows a very restricted distribution especially when compared with other leucine-rich repeat proteins (12–14).

A number of proteases have been suggested to contribute to the degenerative process in the IVD, including matrix metallo-
proteinases (MMPs), aggrecanases, and cathepsins. MMP-1, -2, -3, -7, -8, -9, and -13 along with ADAMTS-4 and -5 have been shown to be up-regulated in the IVD during degeneration and are responsible for the breakdown of important components of the extracellular matrix, including aggrecan and collagen (15, 16). Furthermore, striking expression of cathepsins K, D, and L has been shown in degenerate IVD tissue (17). However, many of the aforementioned proteases also have significant roles in normal matrix remodeling (18). MMP proteolysis of aggrecan has been demonstrated to be mainly a process of normal turnover in the disc, and evidence suggests that cathepsin K also has a significant role in normal turnover (19). HTRA1 is a serine protease initially described in bacteria (20). Several substrates have been described for HTRA1, including fibronectin, type II collagen, decorin, aggrecan, elastin, bone sialoprotein, and matrix Gla protein (21). Mounting evidence suggests that it plays a central role in the pathology of arthritic diseases, such as osteoarthritis (22, 23), and in the degradation of articular cartilage (24). Elevated levels of HTRA1 have also been found in degenerate IVD tissue (25). Other members of the HTRA family have not yet been described in the disc (21). However, in osteoarthritic cartilage, elevated levels of HTRA3 have been reported. HTRA3 has been demonstrated to have substrates similar to those of HTRA1 in articular cartilage (21).

In this study, we show that CHAD fragmentation is associated with disc degeneration present both in the adult with DDD and the adolescent with AIS. We confirm that fragmentation is created by cleavage at the same site within both disorders and identify HTRA1 as a protease able in tissue to generate the fragment found in degenerate IVD tissue.

**EXPERIMENTAL PROCEDURES**

**Materials**—The horseradish peroxidase (HRP)-conjugated anti-rabbit IgG was from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescence (ECL) detection system was purchased from GE Healthcare. The HTRA1 antibody was purchased from Abcam (Toronto, Canada). The aggrecan neoepitope antibody for the HTRA1 cleavage site was a kind gift from Dr. Zhiyou Yang from the Inflammation and Remodeling Research Unit at Pfizer in Massachusetts. Keratanase II and chondroitinase ABC were purchased from BioLynx Inc. (Brockville, Canada) and MP Biomedicals Inc. (Solon, OH), respectively. The Complete® EDTA-free protease inhibitor mixture tablets were purchased from Roche Applied Science. Coomassie Blue stain was purchased from Bio-Rad. Bovine serum albumin (BSA) was purchased from Sigma-Aldrich. The activated keyhole limpet hemocyanin was purchased from Pierce. Matrix metalloproteinases and aggrecanases were all purchased from R&D Systems (Minneapolis, MN). Recombinant human HTRA1 was purchased from Thermo Scientific (Waltham, MA). Recombinant cathepsins K, B, and L were produced in the yeast *Pichia pastoris* as described (26). The polyclonal rabbit antibody recognizing CHAD was raised against the second disulfide-bonded C-terminal loop. It has been tested for specificity by evaluating cross-reactivity with other proteins. It only stains one band corresponding to CHAD in extracts of human articular cartilage.

**Antibody Production**—A polyclonal antiserum was generated against the peptide YLYLSGGC, which was synthesized by CanPeptide (Pointe-Claire, Canada). The peptide corresponds to a 5-residue sequence from CHAD (YLYLS) with a C-terminal linker sequence (GGC) used for coupling 4 mg of peptide to 4 mg of activated keyhole limpet hemocyanin in accordance with the manufacturer’s instructions. Immunization of rabbits with the coupled peptide for antiserum production was performed by the Comparative Medicine and Animal Resources Centre at McGill University.

**Tissue Source**—Normal adult and juvenile human disc samples were obtained through the Transplant Quebec Organ Donation Program from individuals who had undergone sustained brain death. Samples were harvested within 5 h post mortem. Degenerate disc samples were obtained from consenting adolescents undergoing discectomy and interbody fusion for discogenic axial low back pain without radiculopathy and from adolescent patients with AIS undergoing discectomy to obtain anterior release before correction of spinal deformities. The study was approved by the ethics review board at the Montreal General Hospital, Quebec, Canada.

**Analysis of CHAD Fragmentation**—Disc tissue was finely diced, and proteins were extracted at 4 °C under continuous agitation for 48 h using 15 volumes of 4 M guanidine hydrochloride, 50 mM sodium acetate, pH 5.8, 10 mM EDTA, protease inhibitors. The extracts were separated from the tissue residue by centrifugation. Aliquots of 8 µl of disc extract were prepared for SDS-PAGE by precipitation using 9 volumes of 100% ethanol. Precipitated protein samples were recovered by centrifugation. To ensure that complete precipitation was achieved, the supernatant was dialyzed, concentrated, and analyzed by Western blotting in the same way as the precipitated protein samples. No CHAD was identified in the supernatant, indicating complete precipitation. Pellets were washed once each with 75% ethanol and 95% ethanol before being lyophilized and redissolved in 25 µl of 50 mM sodium acetate, pH 6.0. This was then digested with keratanase II at 1 milliunit/25 µl of extract for 6 h. The solution was then adjusted to 100 mM Tris, 100 mM sodium acetate, pH 7.3 and digested overnight with chondroitinase ABC at 50 milliunits/25 µl of extract. Sample buffer was added directly after digestions, and the proteins were fractionated on 12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes by electroblotting (27). Membranes were blocked with 1.5% (w/v) skim milk powder in 0.01 M Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.6. Antisera were diluted 1:1000 in the same buffer containing 3% BSA. Immunoblotting was performed using antibodies raised against intact CHAD or the CHAD peptide YLYLS corresponding to a disc-specific cleavage site. Bound antibodies were detected by chemiluminescence using the ECL system after incubation with a secondary antibody conjugated to horseradish peroxidase using an LAS4000 Image analyzer (GE Healthcare).

**Ratio Analysis of Fragmented to Intact CHAD**—Band intensity was analyzed on immunoblots using ImageQuant TL software. A ratio was calculated for the intensity of the area representing fragmented CHAD versus the intensity of the area representing intact CHAD. Background intensity was subtracted from all samples. Quantification of CHAD fragment to
intact CHAD ratios in 15 non-degenerate (average age, 45 years; age range, 26–60 years old) and 14 degenerate (average age, 45.3 years; age range, 15–70 years old) tissue donors was performed using this method. Statistical analysis was performed using unpaired t test.

**Analysis of HTRA1 Protein Levels**—Proteins were extracted, and immunoblotting was performed as described under “Analysis of CHAD Fragmentation.” Equivalent amounts of protein were loaded in each sample well. Samples were probed using an HTRA1 antiserum diluted 1:250 in accordance with the manufacturer’s instructions.

**Analysis of HTRA1 Cleavage Site in Aggrecan**—Proteins were extracted and treated with keratanase II and chondroitinase ABC as described elsewhere (10). Bound enzymes were identified by reversed phase liquid chromatography on line with electrospray-ion trap mass spectrometry (MS) (29).

**Protease Digestion**—3 mg portions of human disc tissue (from a donor 13 years of age) were digested with the following proteases: MMP-3 (0.46 mg/ml), MMP-7 (0.43 mg/ml), MMP-12 (0.31 mg/ml), MMP-13 (0.50 mg/ml), ADAMTS-4 (0.44 mg/ml), ADAMTS-5 (0.43 mg/ml), and HTRA1 (0.2 mg/ml). The digestions were performed in 50 mM Tris-HCl, 200 mM NaCl, 5 mM CaCl₂, 0.01% Triton X-100. 1.8 μg of enzyme was added, and the tissue was digested overnight at 37 °C. For HTRA1, 1.8 μg of enzyme was added, and the tissue was digested for 8 h at 37 °C, and then a further 1.8 μg of enzyme was added, and the reaction was continued overnight. For cathepsins, 3 mg portions of human disc tissue were digested in 2.5 mM DTT, 0.15% chondroitin sulfate A, 0.1 M sodium acetate, pH 5.5, 1 mM EDTA overnight at 37 °C with 2.5 μg of cathepsin K (0.09 mg/ml), cathepsin B (0.10 mg/ml), or cathepsin L (0.13 mg/ml).

**RESULTS**

**CHAD in Non-degenerate IVD**—CHAD fragmentation has been described previously in some IVD tissue from adolescents with AIS where the level of fragmentation appeared to correlate with disc degeneration (4). To evaluate whether CHAD fragmentation is absent in normal disc tissue throughout life, non-degenerate disc tissue from donors aged 13, 40, and 60 years of age was analyzed. Protein extracts from the nucleus pulposus and annulus fibrosus of these individuals were analyzed by SDS-PAGE and immunoblotting. CHAD was found to be present and intact in all donors regardless of age (Fig. 1A). Furthermore, intact CHAD was present at similar levels in both nucleus pulposus and annulus fibrosus in all three donors, demonstrating that CHAD is found throughout the disc.

Multiple non-degenerate discs from a 60-year-old donor were then analyzed to determine whether CHAD remained intact at all disc levels in the same individual. All discs between T10–11 and L4–5 showed the presence of only intact CHAD in both the nucleus pulposus and the annulus fibrosus regions, confirming that CHAD remains unfragmented in all non-degenerate discs irrespective of level (Fig. 1B). Thus, in both nucleus pulposus and annulus fibrosus regions of the IVD, CHAD fragmentation is absent from non-degenerate discs regardless of age or level.

**CHAD Fragmentation in Degenerate IVD**—To determine whether CHAD fragmentation is common to different types of disc degeneration, protein extracts from degenerate and non-degenerate discs were compared by SDS-PAGE and immunoblotting. Fragmentation of CHAD was observed in surgically excised discs from adult patients with degenerative disc disease (28-kDa fragment) compared with only intact CHAD in the non-degenerate adult discs (Fig. 2A). Similarly, fragmentation of CHAD was particularly observed in the protein extracts from degenerate discs of patients with AIS, whereas the macroscopically normal AIS discs showed traces of the fragment, probably
indicating that degeneration had already started (Fig. 2B). When compared with each other, the CHAD fragment appears to possess a similar size in both the adult degenerate discs and the degenerate AIS discs. This suggests that the cleavage site responsible for CHAD fragmentation may be common in these two conditions. Furthermore, when comparing 15 non-degenerate and 14 degenerate samples, the ratio of fragmented to intact CHAD was significantly higher ($p = 0.007$) in the degenerate samples (Fig. 2C).

**Correlation of CHAD Fragmentation with Severity of Degeneration**—To further establish CHAD fragmentation as a marker of disc degeneration, the correlation between the level of CHAD fragmentation and severity of degeneration was studied. Punch biopsies were taken from the same degenerate disc at three different sites: macroscopically normal looking tissue, mildly degenerate tissue, and severely degenerate tissue. Upon SDS-PAGE and immunoblotting analysis, CHAD was found to have little fragmentation at the macroscopically normal looking site, a small amount of fragmentation was found at the mildly degenerate site, and a high degree of fragmentation was found at the severely degenerate site (Fig. 3). Thus, with increasing degeneration, the higher the abundance of CHAD fragmentation becomes. Furthermore, irrespective of the degree of degeneration, the size of the CHAD fragment appeared constant.

**Identification of the Cleavage Site of CHAD**—Analysis of the cleavage site at which CHAD fragmentation occurs is necessary to compare the identity of the fragments observed in adults with DDD and adolescents with AIS. For this purpose, tissue extract from a degenerate disc was fractionated by CsCl density gradient centrifugation followed by ion exchange chromatography. The CHAD-containing fractions were pooled, and the proteins were separated by SDS-PAGE. Gel slices were excised in the area of the gel where the fragment was present, and mass spectrometric analysis was performed following trypsin digestion. A peptide, “YLYLSHNDIR,” with an N terminus not generated by trypsin digestion was identified by tandem MS, resulting in a Mascot MS/MS ion score of 45 ($p = 0.09$) where 12 b- or y-ions were matching. This sequence is found in the third leucine-rich repeat of CHAD (Fig. 4). Cleavage occurs between an isoleucine and a tyrosine and predicts a molecular size of 28 kDa, assuming there is no additional cleavage C-terminal of this site. Furthermore, the size of the fragment generated by cleavage at this site is compatible with the size seen in the degenerated adult discs when analyzed by immunoblotting.

An anti-neoepitope antibody recognizing the CHAD fragment was generated by immunizing rabbits with the peptide “YLYLSGGC” coupled to keyhole limpet hemocyanin. When
the cleavage fragments from degenerate IVDs from both AIS and adult patients were compared by SDS-PAGE and immunoblotting analysis using the anti-neoepitope antibody, the cleavage age was found to be identical in both groups (Fig. 5). Furthermore, the single band seen in both samples when analyzed by the anti-neoepitope antibody suggests that there is no further processing at the C terminus.

Identification of the Protease Capable of Generating the CHAD Fragment in Situ—To identify the protease responsible for CHAD cleavage at the site found in situ, several proteases (MMPs, aggrecanases, and cysteine and serine proteases) known to be up-regulated during disc degeneration were used to digest normal disc tissue (17, 25, 30–32). Digestion with MMP-3, -7, -12, and -13 showed no evidence of CHAD fragments retained in the tissue (Fig. 6A). Similarly, digestions performed with ADAMTS-4 and -5 also did not generate CHAD fragments (Fig. 6B).

Digestions performed with cathepsins B and L showed extensive degradation of CHAD (Fig. 6C), although peptide fragments from these digest were too small to visualize using SDS-PAGE and immunoblotting analysis with the anti-CHAD antibody. In contrast, cathepsin K generated a CHAD fragment large enough to be retained in the gel and similar in size to that generated in situ (Fig. 6C). However, analysis performed using the anti-neoepitope antibody did not detect the fragment, demonstrating that the cleavage site was not the same as that present in situ (data not shown).

Digestions were also performed with the serine protease HTRA1. Extraction of the disc tissue and analysis via SDS-PAGE and immunoblotting demonstrated that CHAD fragments of a similar size compared with the in situ fragment were retained in the tissue (Fig. 7). Anti-neoepitope analysis of the disc tissue extract indicated that the cleavage site generated by HTRA1 was identical to that present in situ (Fig. 7).

Consequently, the ability of HTRA1 to cleave CHAD led us to investigate the abundance of HTRA1 protein in degenerate disc tissue as compared with that in normal disc tissue. Elevated levels of HTRA1 protein were observed in both degenerate adult and adolescent scoliotic samples as compared with a normal disc sample (Fig. 8A). HTRA1 is represented by two bands upon analysis of the immunoblot. The higher band is common to all samples, whereas the lower band is observed only in the degenerate samples.

HTRA1 has also been reported to degrade aggrecan, but the presence of the resulting G1 fragment in degenerate disc tissue has not been described. Therefore, an anti-neoepitope antibody recognizing the fragment was used to investigate its presence in disc tissue. As observed for CHAD, an HTRA1-generated aggrecan fragment was detected in degenerate disc samples but not in normal disc samples (Fig. 8B). A band of higher molecular weight than the expected fragment was also detected in all samples. This may be due to internal epitope recognition in the aggrecanase-generated G1 domain present in this position of the gel.

DISCUSSION

In the present study, we show that CHAD fragmentation is a feature of disc degeneration in both the adult with DDD and in the adolescent with premature degeneration due to AIS. We have demonstrated that the site of cleavage is identical in both conditions and shown that the protease HTRA1 is capable of generating the CHAD cleavage site identified in IVD tissue.

Matrix homeostasis in cartilaginous tissues is maintained by a controlled turnover of the constituent macromolecules. Several proteins, cytokines, and proteases must act in concert in the disc to achieve this (18). A loss of balance, however, between newly synthesized macromolecules and proteases leads to the
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Degenerative events characteristic of DDD. Disc degeneration is associated with an elevation in the expression of proteases, often as a consequence of adverse loading (31, 33). This leads to proteolytic degradation of matrix components, including fragmentation of proteoglycans and matrix proteins (1, 34). We have found that CHAD fragmentation is present in degenerate discs, whereas in non-degenerate discs, CHAD is found intact throughout life (4). The amount of CHAD fragmentation increases with the degree of degeneration seen in the disc. As expected, some variability was found when multiple samples were compared. The degenerate group consists of samples from both organ donors with degenerate discs and surgically removed discs from patients with degenerative disc diseases, and they represent a wide range of degeneration. In discs from organ donors, it is easy to separate degenerate and normal areas. This is not the case in surgical samples. It is also difficult to distinguish true non-degenerate tissue from very mildly degenerate tissue, which may explain the low signal detected in the samples designated to the non-degenerate group.

AIS presents a situation where the spine exhibits lateral curvature and a rotation of the vertebrae, resulting in disc wedging and abnormal loading of the discs. This is associated with premature degeneration already in adolescent patients with the disease (35). Proteolysis of matrix components has been shown in donor tissue from patients with scoliosis (36) along with increased matrix metalloproteinase levels (37). Other typical signs of degeneration often seen in adult disc degeneration (38), such as a disorganized collagen network and cell clustering, are also found in adolescent scoliotic discs. Thus, it is not surprising that CHAD fragmentation has also been observed in scoliotic discs showing signs of degeneration (4). In this study, we show that CHAD is processed at the same site (KQLI↓YLYL) in both DDD and AIS. Adverse loading is a common link between these diseases and provides a potential common mechanism responsible for both degeneration and CHAD fragmentation. In a quantitative proteomics study of protein patterns in various normal cartilage tissues, including disc, fragments of CHAD were observed in only the disc samples from one individual chosen for Western blot comparison with the mass spectrometry data. This finding has now been followed up by Western blot of the disc samples from all five individuals of the original study using the new neoepitope antibody. Corroborating the data presented here, there was no reactivity in any of the disc samples, and the fragments previously observed clearly did not contain the epitope (39).

HTRA1 is a serine protease that is ubiquitously expressed in the human (40). Two forms of HTRA1 have been described: the larger corresponds in size to the intact protein, and the smaller is suggested to represent a proteolytically processed form of HTRA1 (41). The intact form has been reported to be present in all human discs, whereas the processed form is more abundant in degenerate discs (25). This has been confirmed by our study of the HTRA1 protein as a ~50-kDa protein representing the intact form is present in all samples, and a ~42-kDa protein representing the processed form is more abundant only in the degenerate samples. The fact that HTRA1 is found in both degenerate and non-degenerate tissue indicates that factors other than its presence regulate its activity. As there are no known natural inhibitors to HTRA1, another mechanism for regulating enzyme activity is likely. It is possible that proteolytic processing may increase HTRA1 activity and explain why a higher level of this form is found in degenerate tissue where fragmented CHAD is also detected. An alternative explanation could be that the CHAD cleavage site is masked in the normal disc or that CHAD is not the preferred substrate for HTRA1 and that other substrates must be processed before HTRA1 can be degraded. Thus, CHAD would remain intact in normal disc tissue unless a threshold level of extracellular matrix proteolysis was exceeded.

In our study, HTRA1 is the sole protease that had the capacity to cleave CHAD at the site seen in vivo in degenerate disc tissue. In contrast, MMP-3, -7, -12, and -13; ADAMTS-4 and -5; and cathepsins K, B, and L were incapable of generating the CHAD fragment found in vivo. Cathepsin K cleavage resulted in a fragment close in size to the 28-kDa fragment observed in degenerate disc tissue. However, mass spectrometry analysis of fragmented CHAD only revealed one new N terminus in this size range corresponding to cleavage by HTRA1. Thus, there is no evidence for cathepsin K activity in vivo. However, it is likely that these proteases play a role during normal disc turnover associated with development and aging, and their prior action may enhance the efficiency of CHAD cleavage by HTRA1.

Unlike CHAD, many proteins in the disc undergo degradation during normal turnover with accumulation of fragments. Aggrecan is the most extensively studied of these molecules (42, 43). It is found in cleaved and intact forms within the disc throughout life, and there are numerous proteases capable of cleaving aggrecan at different sites. Metalloproteinase-mediated degradation of aggrecan has been associated with disc degeneration (44, 45), but it is difficult to utilize such aggrecan fragmentation in the intervertebral disc as a specific marker as it is not possible to distinguish degradation associated with normal tissue turnover from that associated with degeneration (46). It is possible that cleavage sites also exist within aggrecan that are specific to degeneration; in fact, we observed an
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HTRA1-generated fragment present in samples also showing CHAD fragmentation using the neoepitope antibody described by Chamberland et al. (28). However, further evaluation is needed to verify that it is not produced during normal aging and that this fragment cannot be lost due to subsequent metalloproteinase cleavage. A marker that is characterized by degradation and fragment accumulation during degeneration but that is not present during normal turnover in the disc is preferable to distinguish aging from degeneration.

The fact that CHAD is rather resistant to proteolysis by most proteases implicated in normal tissue turnover provides a potential for the preservation of the HTRA1-generated cleavage fragment. The data presented in this study suggest that CHAD fragmentation can be such a marker with the ability to distinguish between the two processes. At this point, it is not clear whether degeneration and aging are two distinct events or whether degeneration simply represents an up-regulated version of normal aging. HTRA1 has not been implicated in normal tissue turnover and may represent a difference between the two processes. However, it is possible that HTRA1 is present in normal disc but that a threshold of activity must be exceeded to induce degradation to any appreciable extent, particularity in CHAD.

Although the C-terminal fragment of CHAD may be a useful marker of degeneration within the disc, its accumulation within the disc extracellular matrix precludes it from being a useful serum marker for clinical practice. Such accumulation is probably a consequence of the retention of most of the leucine-rich repeats, which allows continued interaction of the fragment with the collagen fibrils. However, such interaction is unlikely for the corresponding N-terminal part of the protein. If this N-terminal fragment is found to be mobile and leaves the disc to enter the blood circulation, it could serve as serum marker.

It is clear that HTRA1 is not the only protease implicated in disc degeneration, and proteases from the MMP, cathepsin, and ADAMTS families that are active in normal turnover could also play a role. However, as CHAD provides an important link between disc cells and the matrix in the healthy tissue, its cleavage by HTRA1 could be a pivotal step in disc degeneration. It is therefore possible that therapeutic tools aimed at inhibiting HTRA1 activity could be of value to slow down progression of disc degeneration while not influencing normal disc turnover. However, it remains to be seen whether or not there would be any adverse biological effects from systemic inhibition or whether targeted inhibition of HTRA1 within the disc is feasible.

REFERENCES

1. Le Maitre, C. L., Pockert, A., Buttle, D. J., Freemont, A. J., and Hoyland, J. A. (2007) Matrix synthesis and degradation in human intervertebral disc degeneration. Biochim. Soc. Trans. 35, 652–655
2. Luoma, K., Riihimäki, H., Luukkonen, R., Raininko, R., Viikari-Juntura, E., and Lamminen, A. (2000) Low back pain in relation to lumbar disc degeneration. Spine 25, 487–492
3. Huy, D., Brooks, P., Blyth, F., and Buchbinder, R. (2010) The epidemiology of low back pain. Best Pract. Res. Clin. Rheumatol. 24, 769–781
4. Haglund, L., Ouelet, J., and Roughley, P. (2009) Variation in chondroadherin abundance and fragmentation in the human sciotic disc. Spine 34, 1513–1518
5. Mansson, B., Wengén, C., Mörgelin, M., Saxne, T., and Heinegård, D. (2001) Association of chondroadherin with collagen type II. J. Biol. Chem. 276, 32883–32888
6. Camper, L., Heinegård, D., and Lundgren-Akerlund, E. (1997) Integrin α2β1 is a receptor for the cartilage matrix protein chondroadherin. J. Cell Biol. 138, 1159–1167
7. Haglund, L., Tillgren, V., Önnerfjord, P., and Heinegård, D. (2013) The C-terminal peptide of chondroadherin modulates cellular activity by selectively binding to heparan sulfate chains. J. Biol. Chem. 288, 995–1008
8. Haglund, L., Tillgren, V., Addis, L., Wengén, C., Recklies, A., and Heinegård, D. (2011) Identification and characterization of the integrin α2β1 binding motif in chondroadherin mediating cell attachment. J. Biol. Chem. 286, 3925–3934
9. Shen, Z., Gantcheva, S., Månsson, B., Heinegård, D., and Sommarin, Y. (1998) CHAD expression changes in skeletal development. Biochem. J. 330, 549–557
10. Larsson, T., Sommarin, Y., Paulsson, M., Antonsson, P., Hedbom, E., Wendel, M., and Heinegård, D. (1991) Cartilage matrix proteins. A basic 36-kDa protein with a restricted distribution to cartilage and bone. J. Biol. Chem. 266, 20428–20433
11. Mizuno, M., Fuijisawa, R., and Kuboki, Y. (1996) Bone chondroadherin promotes attachment of osteoblastic cells to solid-state substrates and shows affinity to collagen. Calcif. Tissue Int. 59, 163–167
12. Oldberg, A., Antonsson, P., Lindblom, K., and Heinegård, D. (1989) A collagen-binding 59-kd protein (fibromodulin) is structurally related to the small interstitial proteoglycans PG-S1 and PG-S2 (decorin). EMBO J. 8, 2601–2604
13. Fisher, L. W., Termine, J. D., and Young, M. F. (1989) Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconsecutive tissue proteins in a variety of species. J. Biol. Chem. 264, 4571–4576
14. Blochberger, T. C., Cornet, P. K., and Hassell, J. R. (1992) Isolation and partial characterization of lumican and decorin from adult chicken corneas. A keratan sulfate-containing isoform of decorin is developmentally downregulated. J. Biol. Chem. 267, 20613–20619
15. Roberts, S., Caterson, B., Menage, J., Evans, E. H., Jaffray, D. C., and Eisenstein, S. M. (2000) Matrix metalloproteinases and aggrecanase: their role in disorders of the human intervertebral disc. Spine 25, 3005–3013
16. Bachmeier, B. E., Nerlich, A., Mittermaier, N., Weiler, C., Lumenta, C., Wuertz, K., and Boos, N. (2009) Matrix metalloproteinase expression levels suggest distinct enzyme activities during lumbar disc herniation and degeneration. Eur. Spine J. 18, 1573–1586
17. Ariga, K., Yonenobu, K., Nakase, T., Kaneko, M., Okuda, S., Uchiyama, Y., and Yoshikawa, H. (2001) Localization of cathepsins D, K, and L in degenerated human intervertebral discs. Spine 26, 2666–2672
18. Struiglics, A., and Hansson, M. (2012) MMP proteolysis of the human extracellular matrix protein aggrecan are mainly a process of normal turnover. Biochem. J. 446, 213–223
19. Gruber, H. E., Ingram, J. A., Hoeschler, G. L., Zinchenko, N., Norton, H. J., and Eisenstein, S. M. (1996) Bone chondroadherin lipoprotein II is a receptor for the cartilage matrix protein chondroadherin. J. Biol. Chem. 271, 599–608
20. Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011) HTRA proteinase-mediated proteolysis in protein quality control. Nat. Rev. Mol. Cell. Biol. 12, 152–162
21. Tiaden, A. N., and Richards, P. J. (2013) The emerging roles of HTRA1 in musculoskeletal disease. Am. J. Pathol. 182, 1482–1488
22. Grau, S., Richards, P. J., Kerr, B., Hughes, C., Caterson, B., Williams, A. S., Junker, U., Jones, S. A., Clausen, T., and Ehrmann, M. (2006) The role of human HtrAI in arthritic disease. J. Biol. Chem. 281, 6124–6129
23. Hu, S. I., Carozza, M., Klein, M., Nantermet, P., Luk, D., and Crowl, R. M. (2009) Association of chondroadherin with collagen type II. J. Biol. Chem. 284, 34406–34412
24. Polur, I., Lee, P. L., Servais, J. M., Xu, L., and Li, Y. (2010) Role of HTRA1, a serine protease, in the progression of articular cartilage degeneration. Histol. Histopathol. 25, 599–608
25. Tiaden, A. N., Klawitter, M., Lux, V., Mirsaidi, A., Bahrenberg, G., Glanz,
Chondroadherin Fragmentation in Disc Degeneration

S. Quero, L. Liesbacher, T. Wuertz, K. Ehrmann, M., and Richards, P. J. (2012) Detrimental role for human high temperature requirement serine protease A1 (HTRAA1) in the pathogenesis of intervertebral disc (IVD) degeneration. J. Biol. Chem. 287, 21335–21345
26. Billington, C. I., Mason, P., Magny, M. C., and Mort, J. S. (2000) The slow-binding inhibition of cathepsin K by its propeptide. Biochem. Biophys. Res. Commun. 276, 924–929
27. Peeroen, M. (1988) Blotting with plate electrodes. Methods Mol. Biol. 3, 395–402
28. Chamberland, A., Wang, E., Jones, A. R., Collins-Racie, L. A., LaVallie, E. R., Huang, Y., Liu, L., Morris, E. A., Flannery, C. R., and Yang, Z. (2009) Identification of a novel HtrA1-susceptible cleavage site in human aggrecan: evidence for the involvement of HtrA1 in aggrecan proteolysis in vivo. J. Biol. Chem. 284, 27352–27359
29. Danfelter, M., Onnerfjord, P., and Heinegård, D. (2007) Fragmentation of proteins in cartilage treated with interleukin-1: specific cleavage of type IX collagen by matrix metalloproteinase 13 releases the NC4 domain. J. Biol. Chem. 282, 36933–36941
30. Le Maitre, C. L., Freemont, A. J., and Hoyland, J. A. (2006) Human disc degeneration is associated with increased MMP 7 expression. Biotechnol. Histochem. 81, 125–131
31. Yurube, T., Nishida, K., Suzuki, T., Kaneyama, S., Zhang, Z., Kakutani, K., Maeno, K., Takada, T., Fujii, M., Kurosaka, M., and Doita, M. (2010) Matrix metalloproteinase (MMP)-3 gene up-regulation in a rat tail compression loading-induced disc degeneration model. J. Orthop. Res. 28, 1026–1032
32. Le Maitre, C. L., Freemont, A. J., and Hoyland, J. A. (2004) Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. J. Pathol. 204, 47–54
33. Lotz, J. C., Colliou, O. K., Chin, J. R., Duncan, N. A., and Liebenberg, E. (1998) Compression-induced degeneration of the intervertebral disc: an in vivo mouse model and finite-element study. Spine 23, 2493–2506
34. Feng, H., Danfelter, M., Stromqvist, B., and Heinegård, D. (2006) Extra-cellular matrix in disc degeneration. J. Bone Joint Surg. Am. 88, Suppl. 2, 25–29
35. Bertram, H., Steck, E., Zimmerman, G., Chen, B., Carstens, C., Nerlich, A., and Richter, W. (2006) Accelerated intervertebral disc degeneration in scoliosis versus physiological ageing develops against a background of enhanced anabolic gene expression. Biochem. Biophys. Res. Commun. 342, 963–972
36. Brown, S., Melrose, J., Caterson, B., Roughley, P., Eisenstein, S. M., and Roberts, S. (2012) A comparative evaluation of the small leucine-rich proteoglycans of pathological human intervertebral discs. Eur. Spine J. 21, Suppl. 2, S154–S159
37. Crean, J. K., Roberts, S., Jaffray, D. C., Eisenstein, S. M., and Duance, V. C. (1997) Matrix metalloproteinases in the human intervertebral disc: role in disc degeneration and scoliosis. Spine 22, 2877–2884
38. Yu, J., Fairbank, J. C., Roberts, S., and Urban, J. P. (2005) The elastic fiber network of the anulus fibrosus of the normal and sciotic human intervertebral disc. Spine 30, 1815–1820
39. Örnerfjord, P., Khabut, A., Reinhold, F. P., Svensson, O., and Heinegård, D. (2012) Quantitative proteomic analysis of eight cartilaginous tissues reveals characteristic differences as well as similarities between subgroups. J. Biol. Chem. 287, 18913–18924
40. Singh, N., Kuppili, R. R., and Bose, K. (2011) The structural basis of mode of activation and functional diversity: a case study with HtrA family of serine proteases. Arch. Biochem. Biophys. 516, 85–96
41. Nie, G., Li, Y., and Salamonsen, L. A. (2005) Serine protease HtrA1 is developmentally regulated in trophoblast and uterine decidual cells during placental formation in the mouse. Dev. Dyn. 233, 1102–1109
42. Sivan, S. S., Tsitron, E., Wachtel, E., Roughley, P. J., Sakkee, N., van der Ham, F., DeGroot, J., Roberts, S., and Maroudas, A. (2006) Aggrecan turn-over in human intervertebral disc as determined by the racemization of aspartic acid. J. Biol. Chem. 281, 13009–13014
43. Johnstone, B., and Bayliss, M. T. (1995) The large proteoglycans of the human intervertebral disc. Changes in their biosynthesis and structure with age, topography, and pathology. Spine 20, 674–684
44. Roughley, P. J., Alini, M., and Antoniou, J. (2002) The role of proteoglycans in aging, degeneration and repair of the intervertebral disc. Biochem. Soc. Trans. 30, 869–874
45. Szrolovicik, R., Alini, M., Roughley, P. J., and Mort, J. S. (1997) Aggrecan degradation in human intervertebral disc and articular cartilage. Biochem. J. 326, 235–241
46. Durigova, M., Nagase, H., Mort, J. S., and Roughley, P. J. (2011) MMPs are less efficient than ADAMTS5 in cleaving aggrecan core protein. Matrix Biol. 30, 145–153