Osteoarthritis and Cartilage 19 (2011) 1254–1262

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Abundance of calpain and aggrecan-cleavage products of calpain in degenerated human intervertebral discs

S. Fukuta †, K. Miyamoto ‡*, K. Suzuki §, H. Maehara †, T. Inoue †, K. Kikuike †, K. Shimizu †

† *Department of Orthopedic Surgery, Gifu University Graduate School of Medicine, Japan*
‡ *Department of Reconstructive Surgery for Spine, Bone and Joint, Gifu University Graduate School of Medicine, Japan*
§ *Division of Orthopedic Surgery, The Japan Baptist Hospital, Japan*

ARTICLE INFO

Article history:
Received 14 September 2010
Accepted 25 July 2011

Keywords:
Disc degeneration
Intervertebral disc
Human
Calpain
Calpastatin
Aggrecanases

SUMMARY

Objective: To assess the expression of calpains and calpain-induced aggrecan fragmentation in early and advanced stages of degeneration of human intervertebral discs (IVDs).
Design: Disc tissue samples of 55 patients (mean age, 51.2 ± 22.3 years) who underwent intervertebral fusion were divided into groups with early and advanced degeneration based on the Thompson magnetic resonance imaging (MRI) scale. In advanced degeneration group, five patients (mean age, 35.5 ± 11.4 years) of lumbar disc herniation (LDH) were included. Protein levels of m- and μ-calpains and their inhibitor calpastatin were assayed, and immunohistochemical techniques were used to localize and quantify the production of the enzymes. To investigate calpain activity, we assayed purified aggrecan fragmentation in disc tissue by Western blotting and immunohistochemistry with VPGVA antibody, which recognizes the m-calpain generated neo-epitope GVA.
Results: Discs at early stages of degeneration expressed low levels of m- and μ-calpains and calpastatin, and few cells expressed degenerative enzymes. At more advanced stages of degeneration, the expression and number of cells immunopositive for m-calpain, μ-calpain and calpastatin were significantly higher. Further finding showed that anti-GVA-reactive aggrecan fragments were significantly higher in discs at advanced compared with early stages of degeneration. Herniated disc samples showed stronger expression and more cells immunopositive for calpains, calpastatin and GVA in the nucleus pulposus than in the annulus fibrous.
Conclusions: The expression of calpains, together with m-calpain-induced degradation products of extracellular matrix, was correlated with the degree of disc degeneration in human IVD tissue. These findings suggest that calpains may be involved in IVD degeneration *via* proteoglycan (PG) cleavage.

Introduction

The causes of low back pain are multifactorial, although, in about 40% of patients, it involves degeneration of the intervertebral discs (IVDs)[1]. During degeneration, the extracellular matrix (ECM) in which the major structural components of IVD can become highly disorganized and denatured[2,3].

Since inflammatory cytokines are present in degenerated disc tissue[4], inflammation is now regarded as a possible initiator of IVD degeneration[5–7]. *In vitro*, inflammatory cytokines have been shown to cause a loss of PGs in the ECM of human disc cells[7,8]. While aggrecan degeneration in the IVD is a marker of the catabolism of matrix components, several families of proteinases including aggrecanases[7,9,10] and matrix metalloporoteinases (MMPs)[11–13] have been found in degraded human IVD. Moreover, specific products of aggrecan cleavage by aggrecanases[9,12] and MMPs[12] have also been detected.

Calpains are a family of calcium-dependent cytosolic cysteine proteinases that catalyze the limited proteolysis of proteins involved in cytoskeletal remodeling and signal transduction, moreover, it has also been implicated in other physiological, structural, mechanical, and molecular changes resulting in a loss of demarcation between the annulus fibrous (AF) and nucleus pulposus (NP). In degenerated IVD, the loss of ECM components exceeds new synthesis, whereas, in normal IVD, the turnover and synthesis of ECM molecules are at equilibrium[9,14,15]. Disc composition changes with age, leading to a state of disc degeneration

* Address correspondence and reprint requests to: Kei Miyamoto, Department of Reconstructive Surgery for Spine, Bone and Joint, Gifu University Graduate School of Medicine, Yanagido 1-1, Gifu City, Gifu 501-1194, Japan. Tel: 81-(0)-58-230-6333; Fax: 81-(0)-58-230-6334.
E-mail address: kei@bg8.so-net.ne.jp (K. Miyamoto).

1063-4584/$ – see front matter
doi:10.1016/j.joca.2011.07.010

resulting from biochemical, genetic inheritance, and environmental factors. Changes in collagen type and decreased proteoglycan (PG) content result in loss of tissue integrity, decreased hydration, and inability to withstand load[14–17], decreasing the viscoelastic properties of the disc matrix[18]. Finally, these degenerative changes contribute to degenerative disc disease, spondylolisthesis, and pathological processes, such as cell cycle regulation, apoptosis, muscular dystrophies, cataractogenesis, and Alzheimer's and Parkinson's diseases[19,20]. The calpains constitute a large family of distinct isozymes that differ in structure and distribution, with the two best characterized members of this family being ubiquitous-μ-calpain (calpain-1) and m-calpain (calpain-2)[21,22]. Further, calpastatin, a specific endogenous protein inhibitor, has been shown to modulate calpain activity *in vivo*[23]. Calpains, which are abundantly present in human articular cartilage, are involved in the proteolysis of cartilage[24,25] and can proteolyze aggrecan under neutral conditions[9,26]. The sites of aggrecan cleavage by m-calpain were found to be at $VPGVA^{709}$-$A^{710}VPVE$[27]. Although the localization of aggrecan fragments proteolyzed by calpains in human articular cartilage has suggested a possible role for calpain in the degradation of cartilage[25], these fragments have not been precisely localized in human IVD. Furthermore, little is known about the association between calpains and IVD degeneration.

Although articular cartilage and IVD have several biochemical properties in common, little is known about the potential function of calpains in IVD degeneration. Direct injection of calpain into rabbit IVDs resulted in histological degeneration, disc height loss, and a significant decrease in uronic acid content[28]. Although neither endogenous calpains in IVD tissue nor calpain-induced degradation products of ECM were demonstrated, the structural changes in rabbit IVD tissue observed after injection of calpains suggests that these enzymes may play a role in IVD degeneration. While many studies have examined the cleavage of aggrecan at aggrecanase sites in articular cartilage, little is known about their contribution to aggrecan degradation in IVDs[10,29].

Given those backgrounds, we hypothesized that if calpain is involved in the degeneration process in human IVDs, calpain, its inhibitor, and calpain cleaved fragments of aggrecan would be present in IVDs. In addition, an increase in cleaved aggrecan products in IVDs at advanced stages of degeneration would suggest a positive role for calpain in the IVD degeneration process. We therefore assessed the levels of expression and the localization of calpains and its inhibitor in human IVDs, at early and advanced stages of degeneration. In addition, in order to further assess the function of calpains in the metabolism of aggrecan in human IVDs, we measured the amounts of GAG-bearing aggrecan products generated by calpains in early and advanced stages of human IVD degeneration.

Materials and methods

Surgical tissue

IVD samples were obtained from patients who underwent spinal fusion operations from April 2007 to May 2009 in our hospital. Informed consent was obtained from patients or their relatives to obtain human tissue samples. The gross morphology of each disc was graded using the Thompson grading scheme (grade I, two patients; grade II, three patients; grade IV, 35 patients; grade V, 10 patients) by MRI T2 imaging[30]. The samples were divided into two groups, those with early degeneration (grades I and II, five patients) and those with advanced degeneration (grades IV and V, 45 patients) (Table I). Samples were also obtained from the lumbar herniated discs of five patients (each grade III) with recurrent intervertebral disc hernia who underwent interbody fusion.

Table I
Data for patient samples

	Early degeneration	Advanced degeneration
No	5	45
Age	12.3 ± 3.5 y.o. (12–16)	63.2 ± 15.1 y.o. (43–85)
Gender	Male: 3, Female: 2	Male: 21, Female: 24
Diagnosis	Idiopathic scoliosis: 5	Spondylolisthesis: 30 DDD: 15
Donor site (spinal level) of disc samples	Lumbar 1/2 ($n = 1$) Lumbar 2/3 ($n = 2$) Lumbar 3/4 ($n = 2$)	Lumbar 1/2 ($n = 2$) Lumbar 2/3 ($n = 8$) Lumbar 3/4 ($n = 11$) Lumbar 4/5 ($n = 14$) Lumbar 5/S ($n = 10$)
Operation	ALIF:5	ALIF:5 TLIF or PLIF:40
Supplement	Herniated disc samples 5 (Male: 3, Female: 2) 35.5 ± 11.4 y.o. (25–41)	

S: Sacral; DDD: Disc degeneration disease; TLIF: Transforaminal interbody fusion; PLIF: Posterior lumbar interbody fusion.

Extracts of NP samples were taken from the center parts and AF samples from the outer parts near the ligament of the IVD. In harvesting NP and AF tissues from degenerated discs, we estimated their positions from the morphology of normal non-degenerated discs. This procedure was performed by a spine surgeon of 10 years experience. Archived blocks of full thickness IVD wedges of 120 degrees from 12 samples (seven from males; three with early and two with advanced degeneration and two with LDH; and five from females, two each with early and advanced degeneration and one with LDH), each incorporating AF and NP, were fixed in 10% neutral buffed formalin and embedded in paraffin. Sections were taken for staining with hematoxylin and eosin.

Chemicals

Rabbit polyclonal antibody against m-calpain (AB1625, Chemicon; Temecula, CA, USA), mouse monoclonal antibody against μ-calpain (MAB3082, Chemicon) and mouse monoclonal antibody against calpastatin (sc-20779, Santa Cruz Biotechnology; Santa Cruz, CA, USA), and mouse monoclonal antibody against β-actin (sc-10731, Santa Cruz) were obtained from their manufacturers. Proteinase free chondroitinase ABC (100332) and keratanases I and II (100810 and 100812) were obtained from Seikagaku Corporation (Tokyo, Japan). Western Lighting Chemiluminescence Reagent Plus was obtained from Perkin Elmer LAS (Boston, MA, USA). Quantitative changes in luminescence were estimated by LAS-1000 UV mini and Multi Gauge Ver.3.0 (Fujifilm, Japan). All other reagents were obtained from standard commercial sources.

Western blot analysis for calpains and calpastatin in IVD tissues

Tissue samples of all patients (AF: 40 mg, NP: 40 mg) (Early: five patients, Advanced: 45 patients, LDH: five patients) were suspended in lysis buffer (300 μl) (1% Nonidet P-40, 0.5% sodium cholate, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 20 mM HEPES, 3 mM $MgCl_2$, 1 mM phenylmethylsufonyl fluoride, 20 μg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM NaF, and 1 mM sodium orthovandate, pH 7.4) and sonicated at 20 kHz three times for 20 s each, with 10 min icing between sonications, using an Astrason XL sonicator (Misonix, New York, USA). Following centrifugation, the protein concentrations of the supernatants were determined using the Bradford protein assay reagent (Bio-Rad). Samples were separated on SDS–polyaclylamidegel electrophoresis (SDS–PAGE) (5–20% gradient gels)

and transferred to polyvinylidene fluoride membranes (PVDF, Millipore). After blocking with 5% fat free milk, the membranes were washed, incubated with primary antibody, washed again, and incubated with secondary antibody. The level of expression was estimated quantitatively by densitometric analysis of the Western blots. The optical density of each target protein was normalized to that of the 45 kDa β-actin band. Each blot shown is representative of three independent experiments. We conducted Western blotting for all 55 patients both AF and NP on each for calpains and calpastatin. Further, immunoblotting of all samples were repeated three times. Data with coefficient variation (CV) of less than 10% of Western blotting was regarded as reproducible.

Immunohistochemical assays of calpains and calpastatin in IVD tissues

Immunohistochemistry was performed on samples obtained from anterior lumbar interbody fusion (ALIF) operations, which were performed on 12 patients: five with idiopathic scoliosis, four with degenerative spondylolisthesis and disc degeneration, and three with disc herniation. Paraffin sections (4 μm) were stained, deparaffinized with xylene and rehydrated through a series of alcohols to phosphate buffed saline (PBS). Endogenous peroxidase activity was blocked by sequential incubation with 0.3% hydrogen peroxidase and 3% bovine serum albumin, each in PBS. Samples were immunoperoxidase stained using the DAKO EnVion System (DAKO Corp., CA, USA), by incubation with primary antibody for 90 min at room temperature. The primary antibodies used were rabbit anti-m-calpain polyclonal antibody (1:100); mouse anti-μ-calpain monoclonal antibody (1:100); and mouse anti-calpastatin monoclonal antibody (1:100). The samples were washed with PBS and incubated overnight at 4°C with labeled polymer horseradish peroxidase (HRP) anti-Rabbit/Mouse. Following thorough washing, the samples were incubated with DAB (3,3′-diaminobenzidine) for 10 min. The samples were washed, dehydrated, and mounted in Pertex. Figure plates were prepared using Adobe Photoshop (Adobe System, Inc., Mountain View, CA). For analysis, each disc was morphologically separated into three areas, with the NP being at the center of the IVD, the inner-AF within 5 mm of the edge of AF, and the outer-AF at up to 5 mm from the edge of the ligament. Within each area, 200 cells were counted and the number of immunopositive cells was determined[6]. Numbers of immunopositive cells were counted in three different areas of each sample and CV among the three values obtained. The results showed that the CVs were less than 20% (11% in the early NP, 17% in the advanced NP, 15% in the LDH-NP, 17% in the early IAF, 18% in the advanced IAF, and 19% in the LDH-IAF), consequently showing that our method for counting cells provides relatively reproducible number of immunopositive cells in each condition. Data were plotted as the mean ± S.E.M., with 95% confidence intervals. The proportions of immunopositive cells by grade were compared using the Mann–Whitney *U* test.

Aggrecan extraction from discs

For all patients samples, PGs within IVD samples were extracted at 4°C for 48 h with 4 M guanidine hydrochloride (GuHCl) in 100 mM sodium acetate, pH 6.8, 10 mM EDTA, 100 mM 6-aminohexanoic acid, 10 mM benzamidine HCl, 10 mM *N*-etylmaleimide and 1 mM phenylmethyl sulphonyl fluoride[31]. After dialysis against 50 mM sodium acetate, pH 6.0, plus protease inhibitors (10 mM EDTA, 100 mM 6-aminohexanoic acid, 5 mM benzamidine HCL, 1 mM PMSF), each sample was adjusted with solid GuHCl to a GuHCl concentration of 0.5 M and its density was adjusted to 1.6 g/ml by adding solid cesium chloride. The samples were centrifuged at 71,500×*g* for 72 h at 10°C. The resulting gradients were fractionated into four equal parts to yield fractions A1–A4 from the bottom of the tubes. Then A1 fraction was adjusted to dissociative conditions by addition of solid GuHCl to 4 M and the density was adjusted to 1.47 g/ml by addition of solid cesium chloride and centrifuged at 71,500×*g* for 48 h at 10°C. The resulting gradients were fractionated into four equal parts to yield fractions A1D1–A1D4 from the bottom of the tubes. The samples were dialyzed at 4°C against 0.05 M sodium acetate pH 6.8 with protease inhibitors, and finally dialyzed against distilled water. The samples were freeze-dried and stored at −80°C.

Detection of aggrecan lytic activity

In a preliminary investigation, we tested A1D1, A1D2, A1D3 and A1D4 from human disc samples against anti-GVA antibody, showing that the intensity of the bands was consistent A1D1 > A1D2 > A1D3 > A1D4 (data not shown). We therefore tested the A1D1 fraction in this study. We also examined these fractions after guanidinium dialysis. The freeze-dried A1D1 samples were dissolved in 50 mM sodium acetate, 50 mM Tris, 10 mM EDTA, pH 7.6, and sulphated gylcosaminoglycans were assayed by the 1,9-dimethylmethylene blue assay[32]. For deglycosylation, samples in solution were incubated with protein free chondroitinase ABC (0.1 U/100 μg GAG) for 2 h at 37°C, adjusted to 1 mM PMSF and 10 mM *N*-etylmaleimide and incubated with keratanases I (0.1 U/100 μg GAG) and II (0.01 U/100 μg GAG) for 1 h[33]. The deglycosylated PGs were separated on 4–12% SDS–PAGE gels (Super Sep ace™, Wako, Japan) and transferred to membranes using the wet transfer method. The membranes were incubated with VPGVA antibody (1:3000), which recognizes the aggrecan cleavaged by m-calpain followed by incubation with anti-rabbit-IgG (1:3000, Dako, P0448). Western Lighting Plus-ECL (Perkin Elmer, Waltham, USA) was used. VPGVA antibody was used in our *in situ* study of articular cartilage[25]. Western blotting was conducted for all 55 patients both AF and NP for GVA. Further, immunoblotting of all samples were repeated three times.

Statistical analysis

Student's *t* tests were used for comparisons between groups of samples from patients with early and advanced stages of disc degeneration. Mann–Whitney tests were used for between group comparisons of the proportion of cells expressing each protein within the NP, inner-AF, and outer-AF areas, and the Wilcoxon paired sample test was used to compare the proportions of immunopositive cells in different areas of the discs (i.e., NP vs inner-AF, NP vs outer-AF, and inner-AF vs outer-AF). This analysis was performed using all disc sections, regardless of level degeneration. Further, *post-hoc* Tukey–Kramer test was used to compare the intensity of protein expression for Western blots of GVA. All statistical analyses were performed using Stat View 5.0 (SAS Institute, Cary, NC).

Results

Expression of calpains and calpastatin in human IVDs

M-calpain, μ-calpain and calpastatin were expressed in the AF and NP areas of both early and advanced degenerated human IVDs. All the samples from IVD tissue demonstrated a clear positive band at 80 kDa, corresponding to the large subunit of m-calpain[34]. In addition, bands at 80 kDa, corresponding to μ-calpain, and at

115 kDa, corresponding to calpastatin, were also observed. [Fig. 1(A)] The expression of all three proteins was higher in samples from advanced degenerated disc than in samples from early degenerated discs. [Fig. 1(B)] There were no differences, however, between the NP and AF areas for early and advanced degenerated discs. Quantitative analysis of Western blotting results showed that the expression of all three proteins, m-calpain, μ-calpain and calpastatin, was significantly higher in advanced than in early degenerated disc samples within both the AF and NP ($P < 0.001$) (Table II).

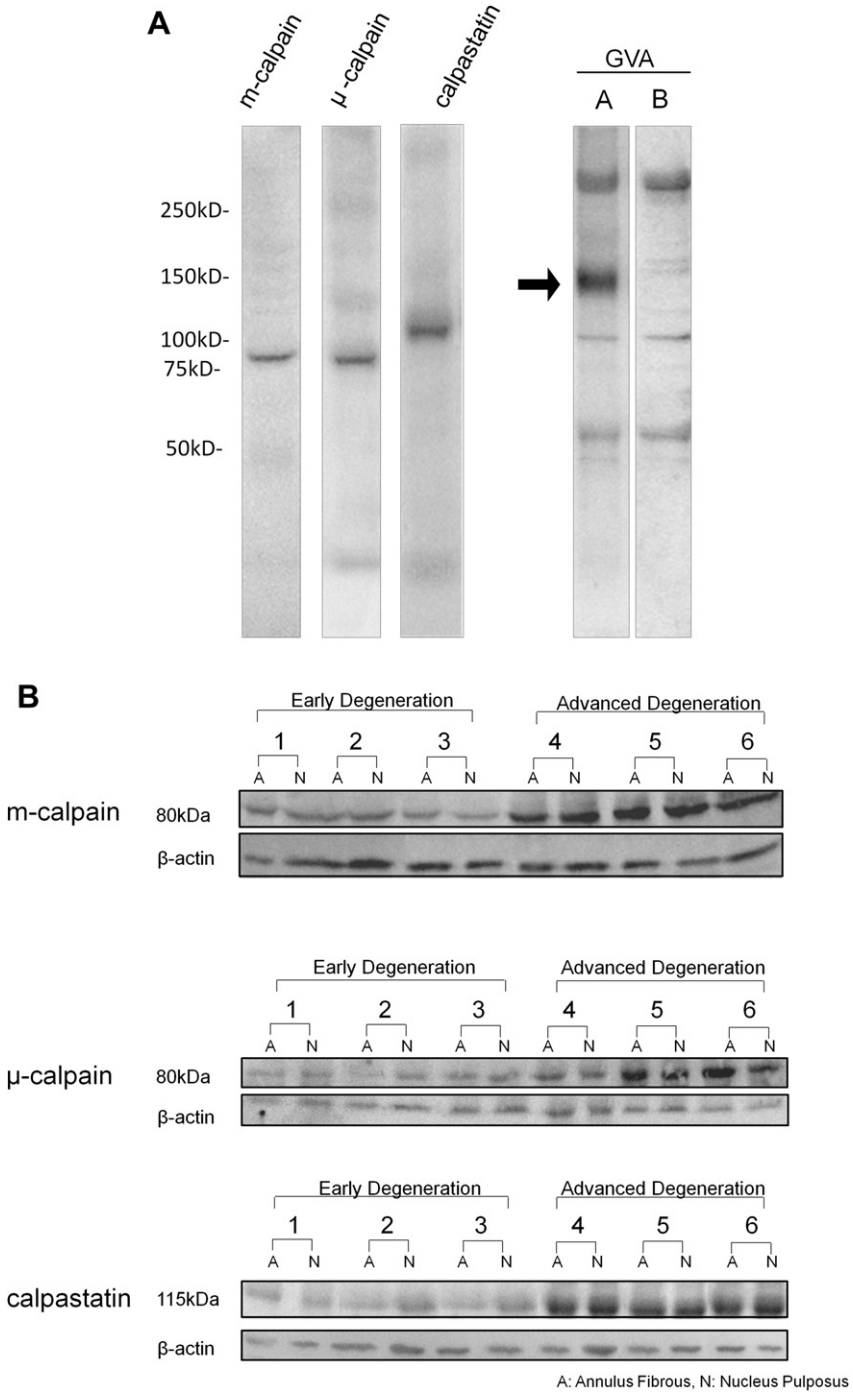


Fig. 1. A: Western blotting with each antibody. Left panel: The molecular masses of m-calpain, μ-calpain, calpastatin (80 kDa, 80 kDa, 115 kDa, respectively). All protein samples from NP were sonicated, with 20 μg protein loaded per lane. **Right panel: Molecular masses positive for GVA (140 kDa)**. Lane A shows blotting of the A1D1 fraction of 20 μg GAG from NP after treatment with the deglycosylation enzymes keratanases I and II and chondroitinase ABC. Lane B shows blotting of the deglycosylation enzymes as a negative control. A band corresponding to GVA was observed in lane A (black arrow). **B: Representative expression of m-calpain, μ-calpain and calpastatin proteins in human IVD tissues at early and advanced stages of degeneration**. Western blot analyses of proteins from the AF and NP areas of human IVD tissue. Results shown are representative of findings in all 55 patients (five early, 50 advanced include five LDH). 20 μg protein were loaded onto each lane. In present figure, Western blots from three early and three advanced degenerated are shown. One bracket (A–N) represents samples from the one patient. Patient number on each bracket is common to each figure in Fig. 1B. Antibodies to m-calpain, μ-calpain, and calpastatin detected single bands of molecular mass 80 kDa, 80 kDa and 115 kDa, respectively.

Table II
Densitometric analysis of Western blotting for m-calpain, μ-calpain and calpastatin/β-actin proteins in human IVD tissues at early and advanced stages of degeneration. (*Mean difference shows difference between Early and Advanced group* (95%CI))

		Early ($n = 5$)	Advanced ($n = 50$ include five LDH)	Mean difference (95%CI)	P value
m-calpain	AF	1.0 ± 0.1	3.2 ± 0.7	2.17 (1.57, 2.76)	<0.001
	NP	0.9 ± 0.1	3.0 ± 0.7	2.05 (1.38, 2.72)	<0.001
μ-calpain	AF	1.0 ± 0.2	3.1 ± 0.8	2.09 (1.40, 2.79)	<0.001
	NP	1.0 ± 0.1	3.0 ± 0.7	1.98 (1.37, 2.59)	<0.001
Calpastatin	AF	1.0 ± 0.1	2.1 ± 0.2	1.06 (0.88, 1.24)	<0.001
	NP	1.1 ± 0.1	2.4 ± 0.2	1.24 (1.06, 1.42)	<0.001

The P values were calculated with Student's t test.
n: number of patients.

Immunohistochemical localization of calpains and calpastatin and the proportion of immunopositive cells for each

Using antibodies against m-calpain, we observed nuclear and cytoplasmic staining (arrows) of disc cells in the AF and NP of both early and advanced degenerated samples, with the percent positive cells being higher in advanced than in early degenerated samples. The location and ratio of immunopositive cells were similar in LDH as in advanced degenerated samples. Immunopositive tendencies were also detected for both μ-calpain and calpastatin (Fig. 2). Low proportions of cells in the NP and inner-AF areas of early degenerated samples were positive for m-calpain, μ-calpain and calpastatin. In NP areas of advanced degenerated samples, however, 72 ± 10% [mean ± standard deviation (SD)] of the cells stained positive for m-calpain, 63 ± 16% for μ-calpain, and 52 ± 12% for calpastatin. In the inner-AF areas of advanced degenerated samples, 58 ± 11% of the cells stained positive for m-calpain, 49 ± 12% for μ-calpain, and 43 ± 10% for calpastatin. In NP areas of herniated disc samples, 73 ± 7.3% of the cells stained positive for m-calpain, 52 ± 10% for μ-calpain, and 58 ± 9.0% for calpastatin. These findings indicate that the proportions of cells in the NP and inner-AF of advanced degenerated and in the NP of LDH samples positive for the calpains and calpastatin increased significantly as degeneration or herniation became more severe ($P < 0.05$) (Table IV). On the other hand, there were no significant differences in the proportion of cells positive for m-calpain, μ-calpain and calpastatin in the outer-AF areas. However, there were no significant differences in the proportion of AF cells positive for calpains and calpastatin between LDH tissue and early degenerated tissue (Table IV). All IgG controls were negative.

Aggrecanolytic activity of m-calpain in IVD tissue

M-calpain proteinase-generated fragments were detected using GVA antibody. Western blot analyses using anti-VPGVA antibody showed that a specific 140 kDa band was present in each sample from early and advanced degenerated tissue sample and in the AF and NP areas of each. (Fig. 3) Level of GVA fragments was significantly higher in tissues from patient with advanced than with early degeneration ($P < 0.001$). Further, staining was significantly stronger in severe degeneration grade V samples than in mild degeneration grade IV samples ($P < 0.05$). Level of GVA fragments was similar in the NP and AF areas of advanced degeneration samples, but was significantly higher in the NP than in the AF areas of herniated discs ($P = 0.001$) (Table III). In immunohistochemistry, using polyclonal antibody against GVA, we detected nuclear and cytoplasmic immunopositivity (arrows) in both the AF and NP of early and advanced degenerated samples. Similar findings were observed for the calpains and calpastatin (Fig. 4).

Proportion of cells immunopositive for GVA

In the NP and inner-AF areas of advanced degenerated samples, 74 ± 13% and 63 ± 13% of the cells, respectively, stained positive for GVA. In the NP and inner-AF areas of herniated disc samples, 79 ± 15% and 54 ± 14% of the cells, respectively, stained positive for GVA. These findings indicate that the proportions of cells in these two areas immunopositive for GVA increased significantly as degeneration or herniation became more severe ($P < 0.05$). Calculations of the coefficients of variation among the results from the three different areas which is less than 20% showed relatively reproducible (Table IV).

Discussion

Overall findings

We have shown here for the first time that both the NP and AF areas of human IVDs contain calpains and calpastatin. In addition, these areas contained the GVA fragment, generated by m-calpain proteolysis. Interestingly, the expression of both the calpains and GVA fragments was significantly higher in IVDs at advanced than at early stages of degeneration. Those findings suggest that the calpains may play a role in IVD degeneration.

Abundance of calpain in degenerated discs

MMPs and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS), enzymes known to initiate articular cartilage degradation, have been observed in both degenerated and herniated IVDs[10,11,35,36]. The calpains may be other candidate proteases involved in the breakdown of articular cartilage in arthritic diseases[25–27,37,38]. While the enzyme ADAMTS4 is involved in the cleavage of aggrecan during human IVD degeneration[9], calpain also may be important for aggrecan cleavage by proteinase. However, the involvement of calpains in ECM breakdown is still unclear. Thus, our finding, of high expression of the calpains and GVA fragments in degenerated IVD tissues, may shed light on the potential function of calpain in the initiation and progression of IVD degeneration.

GVA expression in normal human IVDs

In bovine auricular cartilage, the aggrecan product equivalent to G1-G2-GVA, which is generated by calpains, was detected using an equivalent monoclonal antibody, SK-28[26,37]. This aggrecan product was not found in fetuses, but only in mature samples[37]. In using human IVD tissues, however, we found that G1-G2-GVA was present in IVDs from younger people and in degenerated IVDs. Moreover, the GVA product was detected in samples from both newborns and children[39]. Suggesting that, in addition to its involvement in IVD degeneration, calpain is also involved in the normal aggrecan turnover process in human tissues. We found that the levels of expression of calpains, calpastatin and GVA were significantly higher in the NP and inner-AF than in the outer-AF. Although, the aggrecan content is lower in the outer-AF than in the NP and inner-AF[40], disc degeneration may start within the NP. As the degeneration becomes more severe, the cytoplasm and ECM in the inner and outer-AF regions may become affected[6,12,40].

Changes in GVA and location in degraded discs

We also found that the level of calpain generated GVA was significantly higher in grade V than in grade IV IVDs, suggesting not only the presence of abundant amounts of GVA positive protein in

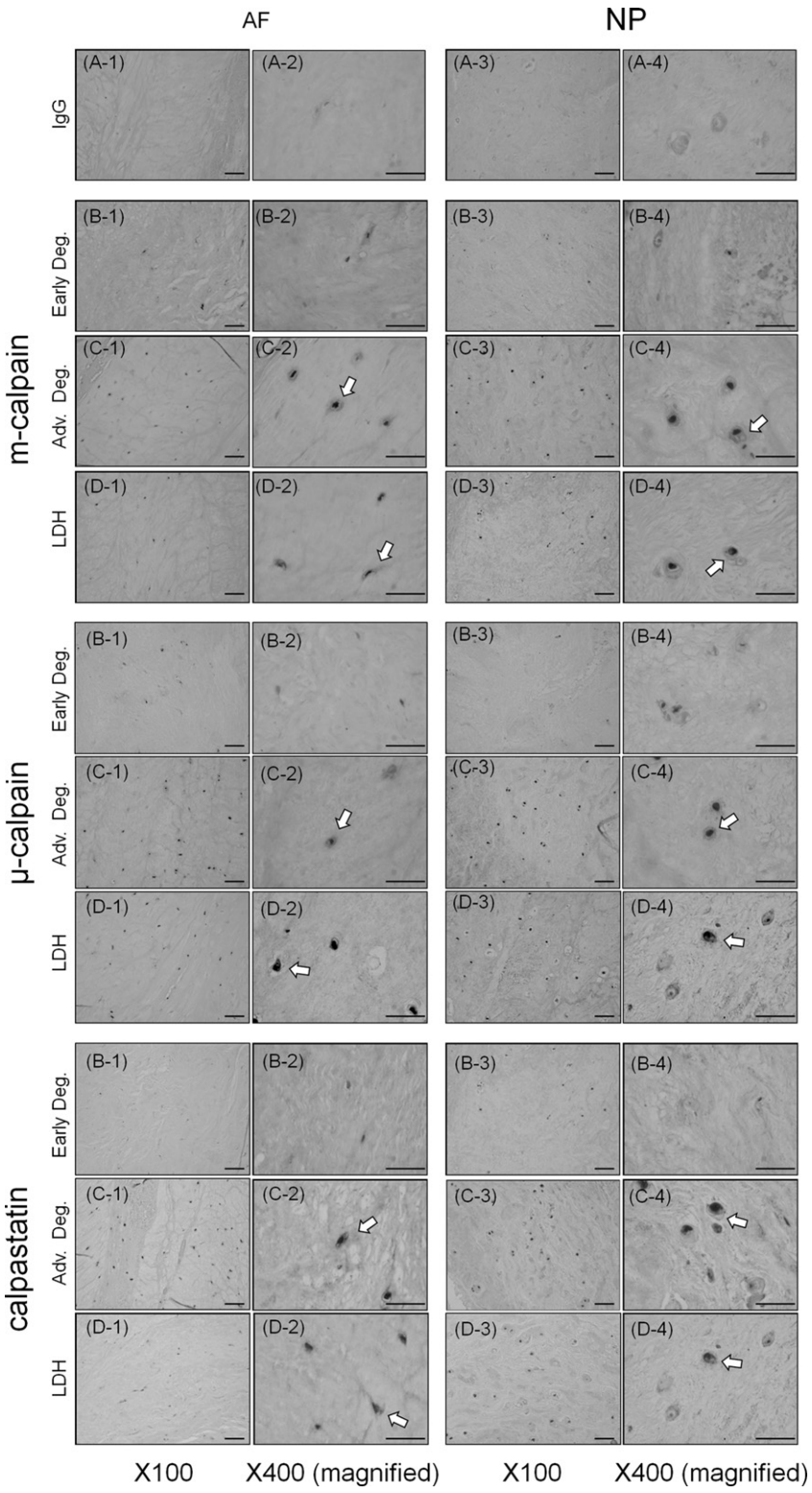


Fig. 2. Representative immunohistochemical analysis of the expression of m-calpain, μ-calpain, and calpastatin in human IVD tissue at early and advanced stages of degeneration. Samples from 12 patients (five early, four advanced, three LDH) were analyzed by immunohistochemistry, with the figure showing representative data from three patients, at early (B) and advanced (C) stages of degeneration and LDH (D). The results in Fig. 2 are from the same patients as WB in Fig. 1B. (A) represents tissue samples incubated with anti-rabbit-IgG. AF (inner-AF) and NP cells, including nuclei, from samples at early and advanced stages of degeneration samples were positive for each antibody. The nuclei, cytoplasm and ECM in the samples at advanced stages of degeneration were also strongly positive (arrows). AF cells in samples at advanced stages of degeneration were more strongly immunoreactive than samples from early stages of degeneration and LDH samples. Additionally, NP cells from samples at advanced stages of degeneration and LDH were strongly immunoreactive to all antibodies. Bars: X100; 200 μm, X400; 50 μm.

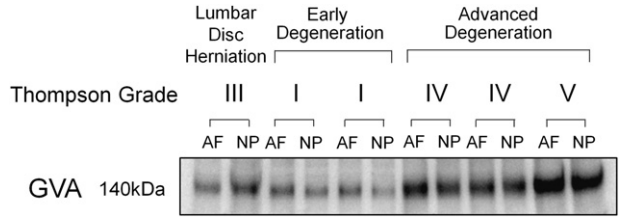


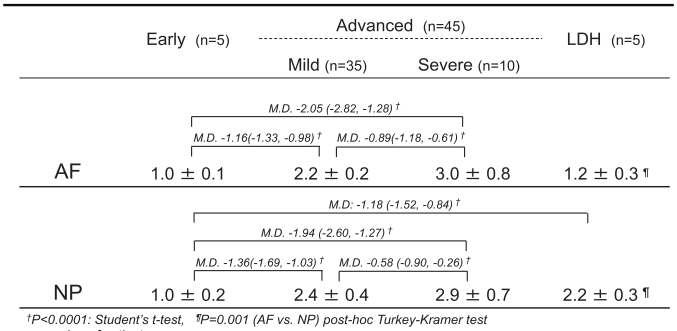
Fig. 3. Representative Western blot analysis of the expression of GVA in human IVD tissue at early and advanced stages of degeneration. Samples from the AF and NP areas of human IVD tissue of 55 patients (five early, 45 advanced, five LDH) were assayed by Western blotting with anti-GVA antibody. 20 μg GAG was loaded onto each lane. The figure shows blotting of samples from six patients (one LDH, two early, three advanced). The brackets (A–N) indicate that these pairs of AF and NP samples are from the same patients. We observed a 140 kDa aggrecanase mediated aggrecan fragment cleaved at the GVA domain in human IVD tissues during both early and advanced stages of degeneration, with higher expression in advanced than in early stage.

severely degraded IVDs but that m-calpain plays a long term disaggregating function in human life. Additionally, the demonstration of both calpains and increased GVA in herniated disc samples suggests that the calpains are involved not only in a gradual degeneration process but also in the acute/subacute process of inflammation. These findings may provide insight into the function of calpains, not only in modulating cellular apoptosis and calcification[28,41], but also on the cleavage of aggrecan, which is involved in maintaining the viscoelastic properties of IVD tissue.

Balance of calpains and calpastatin

Alternations in the levels of expression of calpain and calpastatin, along with differentiation have been reported in kidney and brain tissue samples[23,42,43]. The balance between the calpains and their endogenous inhibitor calpastatin is important in protecting organs from pathological conditions[44]. We think the balance of acceleration; calpain and blake: calpastatin is required to control long term degeneration in IVD to prevent its special function. Many studies have described how calpastatin induces disease progression or degeneration[45,46]. Our results suggest this balance in controlling one aspect of degeneration in human IVD. Similarly, close correlations have been observed between the activities of MMPs and the levels of tissue inhibitor of metalloproteinase (TIMP) in normal cartilage. Moreover, controlling inhibitors have been found to be synthesized in parallel with degenerative enzymes in severely degraded cartilage[47,48] and IVD samples[6,11]. Thus, calpastatin may be involved in preventing calpain activation prior to IVD degeneration. Knowledge of the mechanism of inhibition of calpastatin may

Table III
Densitometric analysis of Western blotting for GVA protein in human IVD tissue from early and advanced stages of degeneration and LDH. (M.D.; Mean Difference shows difference between each groups (95%CI))



†P<0.0001: Student's t-test, ¶P=0.001 (AF vs. NP) post-hoc Turkey-Kramer test
n: number of patients

Table IV
Percentage of immunopositive cells for calpains, calpastatin, and GVA in 3 different segments in intervertebral tissue (mean ± *SD* %)

Antibody	Segment of IVD	Early ($n = 5$)	Advanced ($n = 4$)	*P* value vs Early	LDH ($n = 3$)	*P* value vs Early
m-calpain	OAF	11 ± 3.0	30 ± 13	0.065	24 ± 10	0.071
	IAF	20 ± 3.5	58 ± 11	0.014	35 ± 11	0.092
	NP	17 ± 3.2	72 ± 10	0.014	73 ± 7.3	0.025
μ-calpain	OAF	10 ± 3.1	39 ± 19	0.085	21 ± 6.5	0.051
	IAF	18 ± 9.5	49 ± 12	0.014	20 ± 5.5	0.881
	NP	13 ± 3.3	63 ± 16	0.014	52 ± 10	0.025
Calpastatin	OAF	6.4 ± 2.4	22 ± 10	0.081	11 ± 3.8	0.070
	IAF	12 ± 3.3	43 ± 10	0.014	12 ± 2.5	0.882
	NP	8.1 ± 2.5	52 ± 12	0.014	58 ± 9.0	0.025
GVA	OAF	8.2 ± 2.7	20 ± 9.2	0.108	30 ± 18	0.051
	IAF	19 ± 4.1	63 ± 13	0.014	54 ± 14	0.025
	NP	12 ± 3.0	74 ± 13	0.014	79 ± 15	0.024

OAF: outer-AF, IAF: inner-AF. *P* value: vs early degenerated disc in each group. Mann–Whitney test.

contribute to the future development of therapeutic agents that specifically inhibit the calpains, thus blocking degeneration of IVDs.

Clinical relevance

The results described here may provide an explanation for the mechanism by which calpain induces chemonucleolysis in rabbit IVDs[28]. Following verification of the safety and non-toxicity of calpains, these enzymes can be used for chemonucleolysis, although indications for this therapy are very limited. Further, our findings suggest a future therapeutic approach toward IVD degeneration by regulating the activity of the calpains. While ADAMTS5, one of the aggrecanases, is involved in IVD degeneration[9,49] and injecting the anti-ADAMTS5 oligonucleotide *in vivo* was found to restore degenerated IVDs[49]. Similarly, regulating calpain function in the IVD might be effective in retarding IVD degeneration. Future investigations of the interactions between calpains and

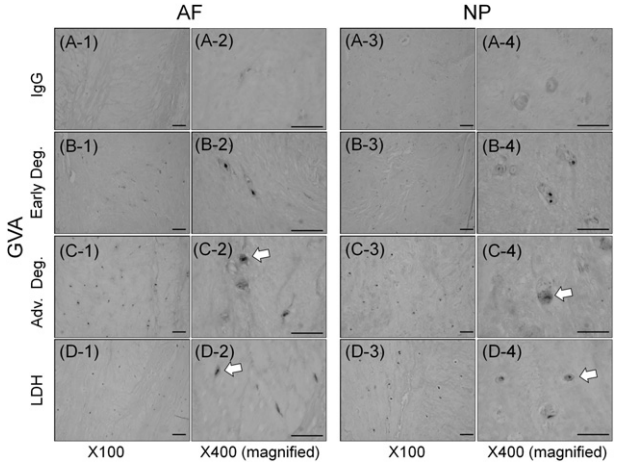


Fig. 4. Representative immunohistochemical analysis of GVA expression in human IVD tissue at early and advanced stages of degeneration. Samples from 12 patients (five early, four advanced, three LDH) were analyzed by immunohistochemistry, with the figure showing representative data from three patients, at early (B) and advanced (C) stages of degeneration and LDH (D). The results in Fig. 4 are from the same patients as WB in Fig. 3. (A) represents tissue samples incubated with anti-rabbit-IgG. AF (inner-AF) and NP cells, including nuclei, from early and advanced degenerated samples, were positive for each antibody. Binding of anti-VPGVA was markedly positive in the nuclei and cytoplasm (arrows) and slightly positive in the ECM of both inner-AF and NP areas. Bars: X100; 200 μm, X400; 50 μm.

aggrecanase, as well as the relative potential of calpain in proteolyzing aggrecans, are necessary.

Limitation of the present study

This study had several limitations. We evaluated relative expression of calpains, calpastatin and GVA by Western blotting. Although several statistically significant differences due to the magnitude of IVD degeneration were observed, methodologically, the assessment was semi-quantified. Future studies should utilize more sophisticated methods enabling quantitative comparisons. In addition, all evaluations in this study were of proteins. Further analyses require assays of m-RNA levels. Second, the mechanisms and pathways involved in regulating calpains and calpastatin were not well analyzed. Further studies are needed to confirm the location of proteolysis induced by calpain, whether in the cytoplasm or ECM. It is also important to determine whether this type of aggrecan processing achieves disc matrix organizational and biochemical properties that promote or compromise tissue function. In the present study, the stages of early and advanced degeneration were classified by Thompson grading using MRI. Moreover, our early degeneration group included both children with "early" degeneration and adults with "mild" degeneration. Since aging promotes histological changes, alterations in ECM, and increases in the numbers of apoptotic cells[50], our "early" group should be subdivided by patient age and analyzed separately in a future study. Our understanding of disc degeneration would therefore be significantly enhanced if the expression of calpains and aggrecan fragments cleaved by calpains is studied together with IVD histology, alterations in matrix metabolism, and cell apoptotic changes. Additionally, pairs of samples from the same patients at early and advanced stages of IVD degeneration may provide significant information. Lastly, we recognize that this study lacks a control group. To determine the potential involvement of calpains in initiating degeneration, strict non-degenerate samples should be studied as controls.

Conclusion

Calpains, together with the degradation products of ECM generated by m-calpain were shown to be present in the NP and AF of human IVD tissue, and could be quantitatively correlated with the magnitude of disc degeneration. These results suggest that this enzyme is involved in catabolic processes that occur during disc degeneration. Calpains may be the focus of therapeutic approaches to treat or at least slow down the degenerative cascade.

Author contributions

All authors have made substantial contributions to all three of sections (1), (2) and (3) below; (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Conflicts of interest

There are no conflicts of interest.

Acknowledgments

The authors thank Hara Akira and Ayako Taguchi for their support with immunohistochemical experiments.

References

1. Luoma K, Riihimaki H, Luukkonen R, Raininko R, Viikari-Juntura E, Lamminen A. Low back pain in relation to lumbar disc degeneration. Spine (Phila Pa 1976) 2000;25:487–92.
2. Rodriguez E, Roughley P. Link protein can retard the degradation of hyaluronan in proteoglycan aggregates. Osteoarthritis Cartilage 2006;14:823–9.
3. Leung VY, Hung SC, Li LC, Wu EX, Luk KD, Chan D, *et al*. Age-related degeneration of lumbar intervertebral discs in rabbits revealed by deuterium oxide-assisted MRI. Osteoarthritis Cartilage 2008;16:1312–8.
4. Weiler C, Nerlich AG, Zipperer J, Bachmeier BE, Boos N. 2002 SSE Award Competition in Basic Science: expression of major matrix metalloproteinases is associated with intervertebral disc degradation and resorption. Eur Spine J 2002;11:308–20.
5. Seguin CA, Pilliar RM, Roughley PJ, Kandel RA. Tumor necrosis factor-alpha modulates matrix production and catabolism in nucleus pulposus tissue. Spine (Phila Pa 1976) 2005;30:1940–8.
6. Le Maitre CL, Freemont AJ, Hoyland JA. Localization of degradative enzymes and their inhibitors in the degenerate human intervertebral disc. J Pathol 2004;204:47–54.
7. Le Maitre CL, Freemont AJ, Hoyland JA. The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration. Arthritis Res Ther 2005;7:R732–45.
8. Miyamoto K, An HS, Sah RL, Akeda K, Okuma M, Otten L, *et al*. Exposure to pulsed low intensity ultrasound stimulates extracellular matrix metabolism of bovine intervertebral disc cells cultured in alginate beads. Spine (Phila Pa 1976) 2005;30:2398–405.
9. Patel KP, Sandy JD, Akeda K, Miyamoto K, Chujo T, An HS, *et al*. Aggrecanases and aggrecanase-generated fragments in the human intervertebral disc at early and advanced stages of disc degeneration. Spine (Phila Pa 1976) 2007;32:2596–603.
10. Pockert AJ, Richardson SM, Le Maitre CL, Lyon M, Deakin JA, Buttle DJ, *et al*. Modified expression of the ADAMTS enzymes and tissue inhibitor of metalloproteinases 3 during human intervertebral disc degeneration. Arthritis Rheum 2009;60:482–91.
11. Roberts S, Caterson B, Menage J, Evans EH, Jaffray DC, Eisenstein SM. Matrix metalloproteinases and aggrecanase: their role in disorders of the human intervertebral disc. Spine (Phila Pa 1976) 2000;25:3005–13.
12. Sztrolovics R, Alini M, Roughley PJ, Mort JS. Aggrecan degradation in human intervertebral disc and articular cartilage. Biochem J 1997;326(Pt 1):235–41.
13. Bachmeier BE, Nerlich A, Mittermaier N, Weiler C, Lumenta C, Wuertz K, *et al*. Matrix metalloproteinase expression levels suggest distinct enzyme roles during lumbar disc herniation and degeneration. Eur Spine J 2009;18:1573–86.
14. Cole TC, Ghosh P, Taylor TK. Variations of the proteoglycans of the canine intervertebral disc with ageing. Biochim Biophys Acta 1986;880:209–19.
15. Lyons G, Eisenstein SM, Sweet MB. Biochemical changes in intervertebral disc degeneration. Biochim Biophys Acta 1981;673:443–53.
16. Roughley PJ, Alini M, Antoniou J. The role of proteoglycans in aging, degeneration and repair of the intervertebral disc. Biochem Soc Trans 2002;30:869–74.
17. Oegema Jr TR. Biochemistry of the intervertebral disc. Clin Sports Med 1993;12:419–39.
18. Miyamoto K, Masuda K, Kim JG, Inoue N, Akeda K, Andersson GB, *et al*. Intradiscal injections of osteogenic protein-1 restore the viscoelastic properties of degenerated intervertebral discs. Spine J 2006;6:692–703.

19. Sorimachi H, Ishiura S, Suzuki K. Structure and physiological function of calpains. Biochem J 1997;328(Pt 3):721–32.
20. Goll DE, Thompson VF, Taylor RG, Zalewska T. Is calpain activity regulated by membranes and autolysis or by calcium and calpastatin? Bioessays 1992;14:549–56.
21. Suzuki K, Tsuji S, Kubota S, Kimura Y, Imahori K. Limited autolysis of Ca2+-activated neutral protease (CANP) changes its sensitivity to Ca2+ ions. J Biochem 1981;90:275–8.
22. Dayton WR. Comparison of low- and high-calcium-requiring forms of the calcium-activated protease with their autocatalytic breakdown products. Biochim Biophys Acta 1982;709: 166–72.
23. Porn-Ares MI, Samali A, Orrenius S. Cleavage of the calpain inhibitor, calpastatin, during apoptosis. Cell Death Differ 1998; 5:1028–33.
24. Suzuki K, Shimizu K, Hamamoto T, Nakagawa Y, Hamakubo T, Yamamuro T. Biochemical demonstration of calpains and calpastatin in osteoarthritic synovial fluid. Arthritis Rheum 1990;33:728–32.
25. Maehara H, Suzuki K, Sasaki T, Oshita H, Wada E, Inoue T, *et al*. G1-G2 aggrecan product that can be generated by M-calpain on truncation at Ala709-Ala710 is present abundantly in human articular cartilage. J Biochem 2007;141:469–77.
26. Lemke AK, Sandy JD, Voigt H, Dreier R, Lee JH, Grodzinsky AJ, *et al*. Interleukin-1alpha treatment of meniscal explants stimulates the production and release of aggrecanase-generated, GAG-substituted aggrecan products and also the release of pre-formed, aggrecanase-generated G1 and m-calpain-generated G1-G2. Cell Tissue Res 2010;340:179–88.
27. Suzuki K, Shimizu K, Hamamoto T, Nakagawa Y, Murachi T, Yamamuro T. Characterization of proteoglycan degradation by calpain. Biochem J 1992;285(Pt 3):857–62.
28. Wakita S, Shimizu K, Suzuki K, Nakagawa Y, Yamamoto S, Yamamuro T. Chemonucleolysis with calpain I in rabbits. Spine (Phila Pa 1976) 1993;18:159–64.
29. Tsuji T, Chiba K, Imabayashi H, Fujita Y, Hosogane N, Okada Y, *et al*. Age-related changes in expression of tissue inhibitor of metalloproteinases-3 associated with transition from the notochordal nucleus pulposus to the fibrocartilaginous nucleus pulposus in rabbit intervertebral disc. Spine (Phila Pa 1976) 2007;32:849–56.
30. Thompson JP, Pearce RH, Schechter MT, Adams ME, Tsang IK, Bishop PB. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine (Phila Pa 1976) 1990;15:411–5.
31. Hascall VC, Kimura JH. Proteoglycans: isolation and characterization. Meth Enzymol 1982;82(Pt A):769–800.
32. Farndale RW, Sayers CA, Barrett AJ. A direct spectrophotometric microassay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 1982;9:247–8.
33. Fosang AJ, Last K, Gardiner P, Jackson DC, Brown L. Development of a cleavage-site-specific monoclonal antibody for detecting metalloproteinase-derived aggrecan fragments: detection of fragments in human synovial fluids. Biochem J 1995;310(Pt 1):337–43.
34. Lane RD, Allan DM, Mellgren RL. A comparison of the intracellular distribution of mu-calpain, m-calpain, and calpastatin in proliferating human A431 cells. Exp Cell Res 1992;203:5–16.
35. Doita M, Kanatani T, Ozaki T, Matsui N, Kurosaka M, Yoshiya S. Influence of macrophage infiltration of herniated disc tissue on the production of matrix metalloproteinases leading to disc resorption. Spine (Phila Pa 1976) 2001;26:1522–7.
36. Shamji MF, Betre H, Kraus VB, Chen J, Chilkoti A, Pichika R, *et al*. Development and characterization of a fusion protein between thermally responsive elastin-like polypeptide and interleukin-1 receptor antagonist: sustained release of a local antiinflammatory therapeutic. Arthritis Rheum 2007;56: 3650–61.
37. Oshita H, Sandy JD, Suzuki K, Akaike A, Bai Y, Sasaki T, *et al*. Mature bovine articular cartilage contains abundant aggrecan that is C-terminally truncated at Ala719-Ala720, a site which is readily cleaved by m-calpain. Biochem J 2004;382:253–9.
38. Yamamoto S, Shimizu K, Suzuki K, Nakagawa Y, Yamamuro T. Calcium-dependent cysteine proteinase (calpain) in human arthritic synovial joints. Arthritis Rheum 1992;35:1309–17.
39. Struglics A, Hansson M. Calpain is involved in C-terminal truncation of human aggrecan. Biochem J 2010;430:531–8.
40. Adams P, Muir H. Qualitative changes with age of proteoglycans of human lumbar discs. Ann Rheum Dis 1976;35:289–96.
41. Goncalves I, Nitulescu M, Saido TC, Dias N, Pedro LM, José Fernandes EF, *et al*. Activation of calpain-1 in human carotid artery atherosclerotic lesions. BMC Cardiovasc Disord 2009;9:26.
42. Averna M, De Tullio R, Salamino F, Minafra R, Pontremoli S, Melloni E. Age-dependent degradation of calpastatin in kidney of hypertensive rats. J Biol Chem 2001;276:38426–32.
43. Averna M, Stifanese R, De Tullio R, Passalacqua M, Defranchi E, Salamino F, *et al*. Regulation of calpain activity in rat brain with altered Ca2+ homeostasis. J Biol Chem 2007;282:2656–65.
44. Hanna RA, Campbell RL, Davies PL. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. Nature 2008;456:U409–78.
45. Paquet-Durand F, Azadi S, Hauck SM, Ueffing M, van Veen T, Ekstrom P. Calpain is activated in degenerating photoreceptors in the rd1 mouse. J Neurochem 2006;96:802–14.
46. Rao MV, Mohan PS, Peterhoff CM, Yang DS, Schmidt SD, Stavrides PH, *et al*. Marked calpastatin (CAST) depletion in Alzheimer's disease accelerates cytoskeleton disruption and neurodegeneration: neuroprotection by CAST overexpression. J Neurosci 2008;28:12241–54.
47. Walter H, Kawashima A, Nebelung W, Neumann W, Roessner A. Immunohistochemical analysis of several proteolytic enzymes as parameters of cartilage degradation. Pathol Res Pract 1998;194:73–81.
48. Murphy G, Reynolds JJ, Werb Z. Biosynthesis of tissue inhibitor of metalloproteinases by human fibroblasts in culture. Stimulation by 12-O-tetradecanoylphorbol 13-acetate and interleukin 1 in parallel with collagenase. J Biol Chem 1985;260: 3079–83.
49. Seki S, Asanuma-Abe Y, Masuda K, Kawaguchi Y, Asanuma K, Muehleman C, *et al*. Effect of small interference RNA (siRNA) for ADAMTS5 on intervertebral disc degeneration in the rabbit anular needle-puncture model. Arthritis Res Ther 2009;11: R166.
50. Gruber HE, Hanley Jr EN. Analysis of aging and degeneration of the human intervertebral disc. Comparison of surgical specimens with normal controls. Spine (Phila Pa 1976) 1998;23:751–7.