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Aggrecanolysis in human osteoarthritis: confocal localization and biochemical characterization of ADAMTS5–hyaluronan complexes in articular cartilages

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Summary

Objective: Human osteoarthritis (OA) is characterized by aggrecanase-mediated depletion of cartilage aggrecan. We have examined the abundance, location and some biochemical properties of the six known aggrecanases (A disintegrin and metalloproteinase with thrombospondin-like motifs 1 (ADAMTS1) 4, 5, 8, 9 and 15) in normal and OA human cartilages.

Methods: Formalin-fixed, ethylenediamine tetraacetic acid (EDTA)-decalcified sections of full-depth cartilage from human OA tibial plateaus and normal control samples were studied by confocal imaging. Probes included specific antibodies to aggrecanases and two aggrecan epitopes, as well as biotinylated hyaluronan binding protein (HABP) for hyaluronan (HA) visualization. Cartilage extracts were analyzed by Western blot for the individual proteinases and aggrecan fragments.

Results: ADAMTS5 was present in association with cells throughout normal cartilage and was markedly increased in OA, particularly in clonal groups in the superficial and transitional zones, where it was predominantly co-localized with HA. Consistent with the confocal analysis, a high molecular weight complex of ADAMTS5 and HA was isolated from human OA cartilage by isotonic salt extraction and chromatography on Superose 6. The complex eluted with an apparent molecular size of about 2×10^6 and contained major ADAMTS5 forms of 150, 60, 40 and 30 kDa. The yield of most forms on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was markedly enhanced by prior digestion of the complex with either *Streptomyces* hyaluronidase or chondroitinase ABC.

Conclusion: ADAMTS5 abundance and distribution in human OA cartilages is consistent with a central role for this enzyme in destructive aggrecanolysis. HA-dependent sequestration of ADAMTS5 in the pericellular matrix may be a mechanism for regulating the activity of this proteinase in human OA cartilage.

Key words: Aggrecanase, Osteoarthritis, ADAMTS5, Cartilage, Hyaluronan, Confocal microscopy, Immunohistochemistry.

Introduction

Osteoarthritis (OA) of the human knee has been evaluated by multiple criteria including pain[1], plain radiograph[2] and delayed gadolinium-enhanced magnetic resonance imaging of cartilage (dGEMRIC)[3]. Total joint replacement surgery provides tissue samples which have been examined by biochemical[4–6], biomechanical[7,8] and standard histopathology[9] methods. More recently, immunohistochemistry (IHC) of proteins and *in situ* hybridization (ISH) of m-RNA[10–12] have added significantly to our understanding of the cellular pathways which are involved. For example, IHC analysis of clonal nests in OA cartilages has shown that these cells contain increased amounts of proteins including RAGE[13], RUNX-2 and matrix metalloproteinase-13 (MMP-13)[14], tenascin[15], annexin VIII[16], caspases 3 and 9[17] and nitrotyrosine, which is indicative of oxidative stress[18]. Phenotypic changes in these cells are further underscored by ISH which reveals increased expression of aggrecan core protein, link protein, osteopontin, collagen type II, Bcl-2, Sox9, MMP-3, MMP-9, and MMP-13[19]. In another study[20], upregulation was observed for MMP-1, MMP-14 (MT1-MMP), A disintegrin and metalloproteinase with thrombospondin-like motis 5 (ADAMTS5), IL-1, TNF-alpha, COL10A1, MMP-9, MMP-13, Indian hedgehog and caspase 3, and a range of pro-apoptotic marker genes[21]. The mechanisms by which such extensive cellular changes come together in uncontrolled tissue destruction in OA remain unclear, although it is reasonable to assume that unchecked proteolytic cascades which fragment the aggrecan and collagen components are central. In this regard it seems likely that one or more of the aggrecanases (ADAMTS1, 4, 5, 8, 9, 15)

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are responsible for destructive aggrecanolysis and one or more of the collagenases (MMP-1, 8, 13, 14) are responsible for destructive collagenolysis[22].

While it is clear from synovial fluid analysis of aggrecan proteolytic products that aggrecanase activity is enhanced at all stages of human OA[23] it is not yet known which ADAMTSs are involved or how this activity is controlled. Localized cartilage destruction suggests that gradients of proteolytic activity are generated within the tissue and an understanding of these events will require detailed analysis of regulatory mechanisms for these proteinases. In addition to transcriptional control[24], there are a range of posttranslational events (pro-domain removal[25], C-terminal truncation[26]) which have been found to modify ADAMTS activity. In addition, the ADAMTSs appear, like their inhibitor tissue inhibitor of metalloproteinases-3 (TIMP-3)[27], to be uniquely tailored for binding to cell-surface and matrix components through the glycosaminoglycan-binding motifs present in the C-terminal domains[28,29]. This may be particularly important in cartilage destruction where glycosaminoglycans such as chondroitin sulfate, keratan sulfate and hyaluronan (HA) represent major matrix components. In this paper we have used confocal immunostaining and biochemical analysis to examine the relationship between the ADAMTS aggrecanases, aggrecan cleavage products and HA in human cartilage tissue obtained at surgery for OA. The results show that ADAMTS5 is uniquely associated with HA in peri- and intercellular locations within clonal groups of cells, which are also the sites of the most intense staining for the terminal aggrecanase product, G1-NITEGE392.

Methods

CHEMICALS AND ANTIBODIES

Paraplast Tissue Embedding medium was from Tyco Healthcare Group (www.kendallhq.com). Histology grade xylene, 37–40% formalin, Superfrost/Plus slides and Harris-modified hematoxylin (containing acetic acid) were from Fisher Scientific. *Streptomyces* hyaluronidase was from Seikagaku Inc., Western blot and confocal imaging supplies were obtained as described[30]. All other chemicals used were of the highest purity available. General details of the histological and immunohistochemical methods have been published[30]. Briefly, antibodies NIT, KNG and VMA (see Table I for details of all antibodies used in this paper) have been previously characterized with respect to specificity and cross-species reactivity by Western blots[5,28,31–38]. Antibodies DLS, FDG, HNE, YNA and HST were raised in rabbits against the ovalbumin-conjugated peptides in collaboration with Affinity BioReagents, Golden, CO. All antibodies used in this study were affinity purified against their cognate peptides on Sulfolink (Pierce, Rockford, IL) and used at concentrations between 1 and 10 μg/ml. On Western analysis, antibody HNE detected both 105 and 68 kDa forms of ADAMTS1 (METH-1) and antibody YNA detected the 98 kDa form of ADAMTS8 (METH-2). Recombinant ADAMTS1 and ADAMTS8 (from transfected 293T cells) were kindly provided by Dr Luisa Iruela-Arispe. Antibody FDG detected both recombinant forms (50–90 kDa) of ADAMTS9 in transfected HEK-293 cells (kindly provided by Dr Suneel Apte, Cleveland Clinic Foundation) and HST detected a single recombinant construct of 60 kDa (TS15-Cat-Dis-TSR1-Cys-FLAG) in transfected EBNA cells (kindly provided by Dr N. Yamaji, Yamanouchi Pharmaceutical Co.).

AGGRECAN WESTERN ANALYSIS

Tibial plateaus were from four total left knee replacement surgeries (73-year male, 69-year female, 75-year female, 82-year female), all diagnosed with OA (tissue, Grades 2–5 on the Osteoarthritis Research Society International (OARSI) scale[9]). Normal femoral condylar cartilage was provided by Dr Peter Roughley (Shriners Hospital, Montreal), and was obtained at autopsy from four individuals (83-year male, 60-year female, 59-year female, 29-year male) with no recorded history of joint disease. Tissues were washed in phosphate-buffered saline (PBS) (containing proteinase inhibitor cocktail) and stored at −80°C. OA cartilages (Grades 2–4 tissue pooled from medial and lateral surfaces) and normal cartilage were washed in ice-cold phosphate-buffered saline (PBS) (+proteinase inhibitors), and 200 mg wet weight of tissue was sliced into 3 mm × 1 mm discs followed by extraction in 4 M guanidine·HCl (+proteinase inhibitors), 50 mM sodium acetate, pH 7.3, purified by G50 and DE52 chromatography as described[5] and deglycosylated core protein from 75 μg dimethylmethylene blue-glycosaminoglycan (DMMB-GAG) was loaded per lane.

ADAMTS WESTERN ANALYSIS

Extracts were prepared from five separate pools of OA cartilage from lateral and medial tibial surfaces taken at total knee arthroplasty. Patients were of both sexes and were between 59 years and 78 years of age at the time of surgery. The tissues (Grades 2–5 on the OARSI scale[9]) were frozen in liquid nitrogen, powdered with a Bessman Tissue Pulverizer, mixed with 50 mM Tris, 100 mM NaCl, pH 7.0 (+proteinase inhibitors) (3 ml/g tissue) and extracted gently at 4°C for 2 h. Extracts were clarified by centrifugation, the supernatant was added to 3 volumes of absolute ethanol, stored at −20°C for 2 h and precipitates were recovered by centrifugation. Pellets were washed with ethanol, and dissolved in 0.1 M ammonium acetate, 4 mM ethylenediamine tetraacetic acid (EDTA), 0.1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF), pH 6.0 (1 ml per g tissue equivalent) for storage at −20°C. For ADAMTS4, 9 and 15 analysis portions (0.1 ml) were dried and run on SDS-PAGE with and without digestion with *Streptomyces* hyaluronidase. For ADAMTS5 analysis, portions (0.5 ml) were dried in a Speedvac, dissolved in 4 M guanidine/100 mM Tris/1 mM EDTA/pH 8.5 and reduced and alkylated with 10 mM dithiothreitol (DTT) (2 h at 60°C) and 40 mM iodoacetic acid (20 h at room temperature (RT)). Samples were fractionated on Superose 6 (1 cm × 25 cm) in 0.5 M Na acetate, pH 6.5 at 12 ml/h collecting 1.5 ml fractions. Material in fractions 4–21 was precipitated by addition of 3 volumes of absolute ethanol and stored at −20°C for 3 h. Pelleted protein was washed with ethanol and dried briefly. Portions were digested for 2 h at 37°C in the presence or absence of *Streptomyces* hyaluronidase or chondroitinase ABC as previously described[5]. The products were solubilized in gel-loading buffer (with and without DTT) and portions (equivalent to about 100 mg wet wt of tissue) were loaded for ADAMTS5 Western analysis.

IHC

OA human tibiae were obtained from total knee arthroplasty as a single piece, approximately 2 cm deep, containing all articular borders and osteophytes. Normal control cartilages were obtained at autopsy from the medial side of lateral femoral condyles as previously described[39] of a

Table I
Summary of antibodies used in this study. The peptides used for immunization have an N-terminal cysteine added for coupling purposes. The residue numbers shown are taken directly from human sequences in www.ncbi.nlm.nih.gov/entrez

Three-letter antibody notation	Protein detected	Human peptide antigen used for immunization	References for antibody characterization
DLS (also JSCDLS)	Aggrecan core	CGGSGVEDLS1411	See Footnote a
NIT (also JSCNIT)	Aggrecan core	CGGNITEGE392	5,28,36
HNE (also JSCHNE)	ADAMTS1	CHNEFSKASFGSGPA642	This paper
VMA (also JSCVMA)	ADAMTS4	CVMAHVDPEEP403	28,38
KNG (also JSCKNG)	ADAMTS5	CKNGYQSDAKGVKTF649	37,42
YNA (also JSCYNA)	ADAMTS8	CYNAYNYTDMDGNLL608	This paper
FDG (also JSCFDG)	ADAMTS9	CFDGKHFNINGLLPN670	This paper
HST (also JSCHST)	ADAMTS15	CHSTNRLTLAVAW603	This paper

33-year male, a 50-year male and a 41-year female. The suitability of femoral cartilage as a normal control in this study is supported by the finding[40] that important cell biological parameters (such as chondrocyte volume, chondrocyte surface area, the number of cells per chondron, the matrix volume per chondron and the number of chondrons per unit volume) are essentially the same at all sites within the human knee joint. All cartilages remained attached to the sub-chondral bone to prevent swelling-induced morphological changes, the specimens were rinsed briefly with sterile saline, placed in neutral-buffered formalin and fixed for a minimum of 4 days. Tissues were suspended in gauze and decalcification performed in 14% (w/v) EDTA/PBS for 7 weeks. Each OA plateau with underlying bone attached was cut into 28 pieces by dissection through the midline and then at 13 equidistant locations from anterior to posterior across the medial and lateral aspects. Individual pieces were decalcified for an additional 4 weeks in EDTA, equilibrated into 70% ethanol, processed and embedded in paraffin.

IHC probes were individually optimized for sensitivity, specificity and reproducibility. Thin (4 micron) sections were deparaffinized as described[30] and incubated in dilute goat serum (1.5% v/v in PBS) for 20 min, prior to exposure to specific IgGs, diluted to 1, 2, 5 and 10 μg/ml with 1.5% (v/v) goat serum for 30 min at RT. IgG solutions were removed, and sections were washed extensively in PBS before incubation at RT for 30 min with 7.5 μg/ml biotinylated goat anti-rabbit IgG (Vector labs). Sections were rinsed in PBS and then treated with 3% hydrogen peroxide (v/v) in tap water for 10 min, rinsed again in PBS, and incubated with horseradish peroxidase (HRP) labeled avidin–biotin complex (Vector labs) for 30 min at RT, washed with PBS and then incubated with 3,3′-diaminobenzidine (DAB) substrate for 30 min. Negative controls (background staining) for each antibody were established by omission of primary antibody, and incubation with nonimmune rabbit IgG at the same concentration and under the same conditions. Specificity was independently established by Western analysis with human, bovine and recombinant antigens and antigenic peptide blocking experiments[5,30,36,41–43].

The sensitivity was determined by titration (0.1–20 μg/ml IgG) and the concentration generating the maximum differential of immune IgG over nonimmune was chosen for subsequent confocal IHC. For aggrecan epitopes, differential reactivity was evident without antigen retrieval. In contrast, ADAMTS4, 5, 9 and 15 protein epitopes in both normal and OA cartilage required antigen retrieval by proteinase K digestion as previously described in detail[30]. Staining with anti-ADAMTS1 and anti-ADAMTS8 antibodies did not generate signals, which could be readily distinguished from nonimmune control on either normal or OA sections either before or after proteinase K treatment, so these enzymes were not further analyzed. It should be noted that proteinase K treatment of cartilage sections also enhanced HA staining, consistent with removal of HA-bound proteins from HA and provision of additional sites for hyaluronan binding protein (HABP) binding. This result is illustrated in Supplementary Fig. 1.

For confocal fluorescent imaging, sections were stained with primary antibodies as described above, washed in PBS for 5 min and then incubated with AlexaFluor568 goat anti-rabbit IgG (1:250) for 1 h at RT. Sections were also co-stained for HA as previously described[30] using an established method[44], in which the G1 domain of bovine aggrecan was purified, modified with the EZ-link Sulfo-NHS-LC-biotinylation kit (Pierce Inc.) and added to sections at a dilution of 5.0 μg/ml PBS for 3 h at RT. Sections were washed in PBS for 5 min and incubated with AlexaFluor488-streptavidin for 1 h at RT. Sections were washed with PBS, incubated with nuclear stain TOTO-3 (T3604, Molecular Probes Inc., diluted 1:500 in PBS), covered with Vectashield mounting medium (Vector Labs) and imaged on a Leica TCS SP2 Confocal Scanning Laser Microscope at a resolution of 1024 × 1024 pixels. Green fluorescence was detected between 498 and 560 nm after excitation at 488 nm (using Ar/Kr laser source), red fluorescence between 572 and 632 nm after excitation at 561 nm (using He/Ne laser source) and blue fluorescence between 649 and 690 nm after excitation at 633 nm (using He/Ne laser source). Image analyses were performed using Leica Confocal Imaging software, including visualization and verification of molecular level co-localized fluorophores as previously reported[45–47].

To confirm specificity of HA staining, sections were treated at 37°C for 20 min with *Streptomyces* hyaluronidase (2 U/ml) and washed extensively in PBS before application of biotinylated G1 domain.

Results

HISTOPATHOLOGY OF HUMAN TIBIA CARTILAGE TAKEN AT JOINT REPLACEMENT SURGERY

More than 30 tibial plateaus from patients with Stage 4 disease[9] (entire joint surface involved) were examined to establish general appearance and to select representative samples for detailed analysis. Most samples retained cartilage on the lateral plateau, where peripheral osteophytes were common. Consistent with commonly observed severe knee OA, many had lost cartilage down to bone across the middle of the medial compartment, but retained some cartilaginous covering near the central ridge. The samples analyzed in detail here (Fig. 1) were from the left knee of

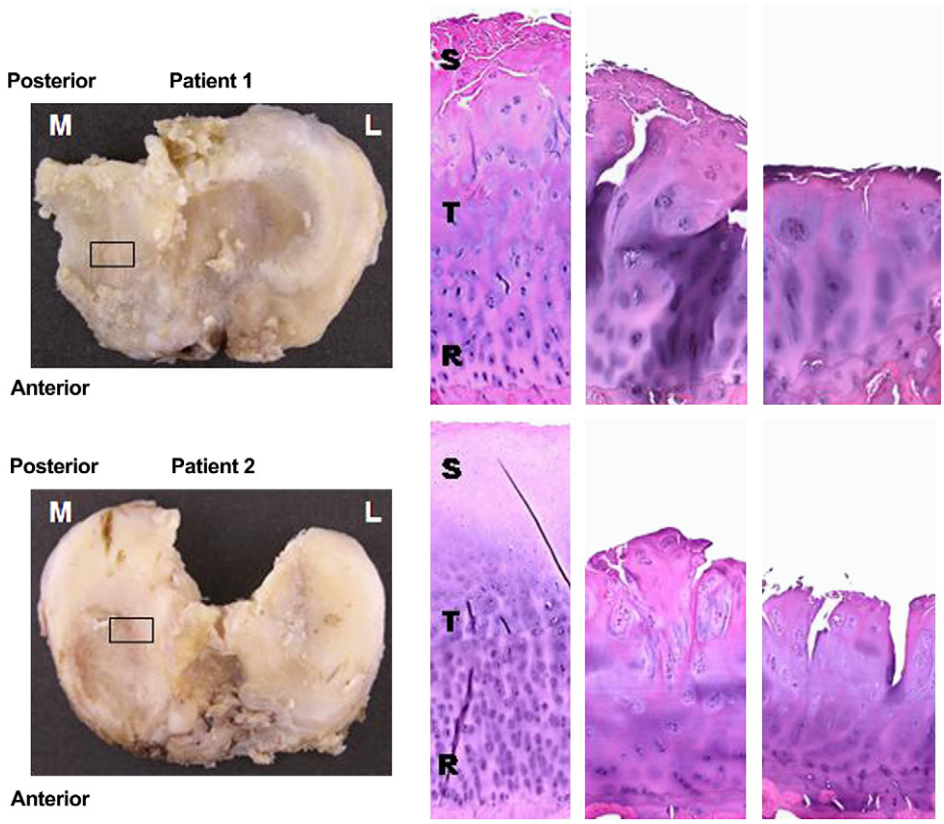

Fig. 1. Appearance and histopathology of tibial plateaus. The gross appearance of tibial plateaus from Patient 1 (58-year female) and Patient 2 (75-year female) are shown with the histological appearance (H and E) of representative samples taken from the area in the black box. The H/E images show the range of histopathology present in this small area. S = superficial zone; R = radial zone; T = transitional zone are defined as described[40]. M = medial; L = lateral. See text for more details.

a 58-year-old obese female (Patient 1, body mass index (BMI) 49) and the right knee of a 75-year-old non-obese female (Patient 2, BMI 29). Patient 1 had bilateral knee OA with no prior surgery and Patient 2 had prior total right hip arthroplasty 4 years earlier.

The 28 paraffin mounts from each patient were sectioned systematically from anterior to posterior to generate mid-block sections, and stained with hematoxylin/eosin (H/E) for histopathological evaluation on the OARSI grading scale[9]. Sections shown were derived from within the small black box shown on the medial aspect and reveal pathologies ranging from Grades 2 to 5 on the OARSI scale[9]. The tibial surfaces from Patient 1 were severely degraded with complete erosion on most of the medial aspect and a large central erosion on the lateral side, with surrounding deep fibrillations. The medial surface was entirely fissured and there were clonal cell nests throughout the depth of the tissue. The tibial surfaces from Patient 2 (lower panels) appeared relatively intact except for a large focal lesion on the anterior medial aspect. Histologically, the smooth appearing surfaces from this patient were rather acellular and were covered with a fibrous layer (left hand, H/E panel). To illustrate how the samples taken from Patients 1 and 2 (Fig. 1) are representative of the whole patient group we have also provided H/E images (10 per patient) from Patients 3–6 (Supplementary Fig. 2) and H/E images (four per patient) from Patients 1–4 (Supplementary Fig. 3).

ANALYSIS OF AGGRECAN SPECIES IN NORMAL AND OA CARTILAGES BY IHC AND WESTERN BLOT

The three-letter notations, peptide epitopes and references to production and characterization of all antibodies used in this study are provided in Table I. To optimize conditions for immunohistochemical analysis of full-depth human cartilages we examined effects of the decalcification method (EDTA and formic acid), the paraffin formulation and slide surface chemistry as previously described[30]. Using these optimized conditions, normal and OA sections were stained with nonimmune IgG, DLS or NIT and examined by light microscopy with DAB visualization (Fig. 2, normal cartilage (top panels), OA cartilages (bottom panels)).

It is clear that some DAB staining was obtained with the 10 μg/ml nonimmune IgG used here. This was seen as a diffuse matrix staining in the superficial zone of the normal cartilage and both matrix and cellular staining in the OA cartilages. However, both immune probes showed an increased intensity and altered distribution of staining which we interpret as specific for the aggrecan epitopes. Thus, in normal tissue DLS staining was seen throughout the full depth of the section, predominantly associated with cells or the cell-associated matrix. NIT staining in normals was variable but largely limited to the cell-associated and interterritorial matrix in the superficial zone. It was also detected with a few cells in the radial zone at the cartilage–bone interface. In OA, DLS staining intensity was generally

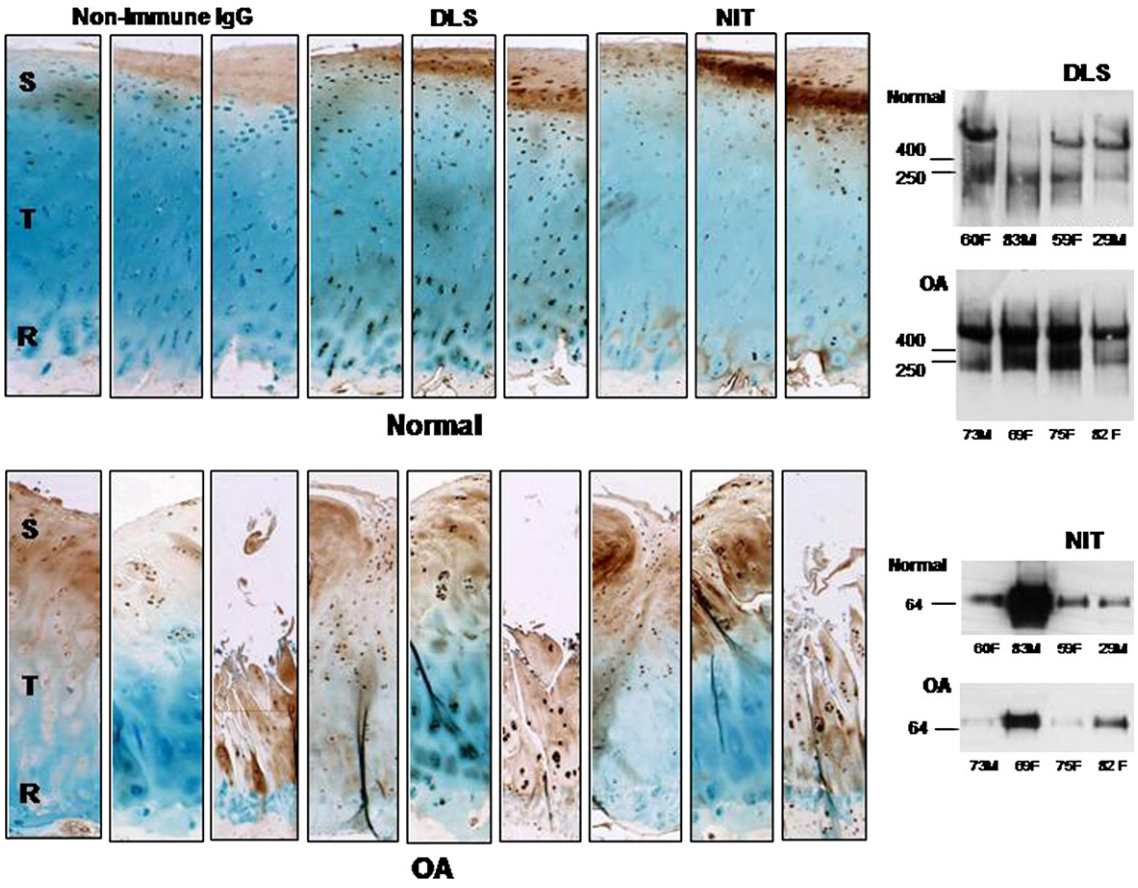

Fig. 2. Immunostaining and Western analysis of normal and OA cartilages for aggrecan epitopes. IHC of normal (41-year female) and OA (Patient 1) cartilages. Shown are three different areas for each patient, representing Grade 1 tissue for the normal and Grades 2, 3.5 and 5, respectively, for the OA patient. A comparison of images obtained with nonimmune IgG (left hand IHC panels), anti-DLS (middle IHC panels) and anti-NIT (right-hand IHC panels) are given. Western analyses of aggrecan epitopes, DLS and NIT, for four normal and four OA patients (tissue, Grades 2–5) are also shown on the right. The bands shown here represent the only DLS- and NIT-reactive species present in these samples. See text and Table I for the explanation of epitope designations (DLS, NIT), sample preparation and histological processing of tissues. The molecular weight markers are for laminin A (400 kDa), myosin (250 kDa) and glutamic dehydrogenase (64 kDa). S = superficial zone; R = radial zone; T = transitional zone.

increased and this was most evident in the intercellular matrix of the transitional zone and in the pericellular matrix of the radial zone. The NIT staining was also clearly increased and more widely distributed in the OA tissue but this was confined to the intercellular matrix in the transitional zone. To illustrate how the samples taken from Patient 1 for analysis of NIT and DLS (Fig. 2) are representative of the whole patient group, we have also provided further NIT staining (four per patient) of Patients 1–4 (Supplementary Fig. 4) and further DLS staining (four per patient) of Patients 1–4 (Supplementary Fig. 5).

As expected from the IHC, Western analysis of aggrecan core species in preparations from four normal and four OA patients (Fig. 2, right side) gave strong signals with both DLS and NIT probes. Other studies with anti-G1 domain antibodies[a] show that DLS reacts with both full-length aggrecan (upper band above the 400 kDa marker) and a major C-terminally truncated aggrecan population (at about 250 kDa) which can be generated by m-calpain digestion and which is a normal constituent of human cartilages (referred to as band a in Refs. 5 and 34). Interestingly, the present data from both Westerns and immunostaining indicate that OA cartilages contain more abundant DLS-reactive products and a higher ratio of full-length molecules to C-terminally truncated molecules, consistent with our data from joint injury patients[5]. The similar, albeit variable, reactivity with NIT in normal and OA cartilage extracts on Western is expected[5], suggesting that the consistently increased staining with NIT in the OA tissue sections (Fig. 2) is due to enhanced antigen accessibility, and not increased accumulation of NIT epitope. The variability of signal between patients on Westerns is probably due to the fact that heterogeneous tissue, which had been pooled from medial and lateral surfaces of the tibia of each patient, was used for extraction (see Methods).

When the normal and OA samples described by IHC in Fig. 2 were analyzed by multichannel fluorescence confocal microscopy (Fig. 3) the data were supportive of the DAB results but much more definitive. Most notably, the nonimmune IgG background staining was essentially undetectable and the immune staining for both DLS and NIT was very clear. In addition the enhanced staining of the aggrecan epitopes in the OA tissue was found at 63× (right-hand panels) to be largely intracellular for DLS whereas NIT was both intracellular and matrix-associated.

[a]Details of the DLS antibody and the human aggrecan species which it detects in human cartilage extracts and synovial fluids are provided in a manuscript in review.[76]

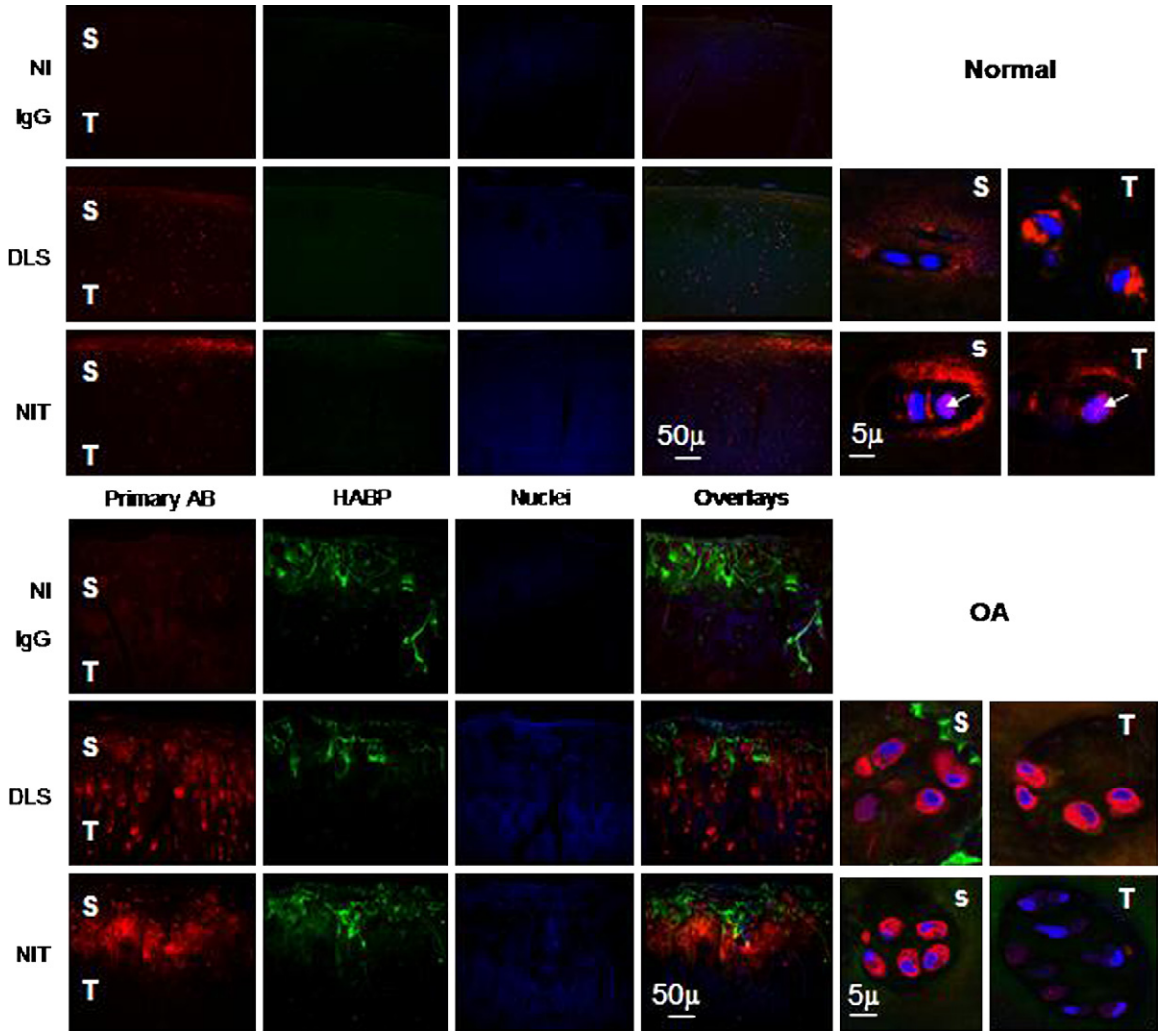

Fig. 3. Confocal imaging of normal and OA cartilage co-stained for aggrecan epitopes and hyaluronan. Contiguous sections of normal cartilage from a 41-year female (top panels) and OA cartilage from Patient 1 (Grade 3.5, bottom panels) were probed with either nonimmune IgG, DLS or NIT as shown, and co-stained for HA and nuclei. Confocal images of individual antibody (Ab) stains for protein (red), HABP for HA (green) and TOTO-3 for nuclei (blue), as well as automated overlays of low magnification (10×) are shown. High magnification images of overlays for DLS and NIT staining in superficial (S) and transitional (T) cell groups are also shown.

Additionally the overlay analysis shows that the HA and aggrecan epitope staining are not co-localized, which would be explained if the HABP probe does not readily detect HA in the presence of high and competing concentrations of cross-linked and HA-bound aggrecan species. The overlay analysis also shows that HA staining can be readily detected in OA cartilage only and in that tissue it is restricted to the superficial and upper transitional zones. This may represent a pool of HA that is readily accessible to the probe due to low concentrations of tissue aggrecan at these degenerating locations (also see Supplementary Fig. 1).

ANALYSIS OF ADAMTSs BY IHC

Attempts to detect ADAMTS proteins in untreated tissue sections, under conditions used for aggrecan probes in IHC (Figs. 2 and 3), were unsuccessful, suggesting that ADAMTS epitopes were masked by other matrix components. Most antigen retrieval methods (heat treatment in Tris or citrate buffers, chondroitinase or hyaluronidase digestions) gave no improvement, however, a mild and controlled digestion with proteinase K enabled specific staining to be studied. This effect is illustrated in Fig. 4 (left panels) where staining of normal cartilage with KNG (ADAMTS5) is shown. When this protocol was applied to OA cartilages (Fig. 4, right panels), some background staining with nonimmune IgG was found, however, marked enhancement was seen in matrix staining for VMA (ADAMTS4) and in cell staining for KNG (ADAMTS5). Indeed, IHC analyses of OA cartilages with KNG showed marked increases in cell-associated staining for ADAMTS5 protein in the superficial and transitional zones of all diseased cartilage sections examined. Moreover, the increased staining was most evident for clonal nests of chondrocytes in the severely degenerate regions of the tissue, whereas minimal or no staining for KNG was seen in the radial zone cells (data not shown). VMA staining was essentially undetectable in normal cartilages (see also Fig. 8) and was enhanced in the matrix of OA tissues.

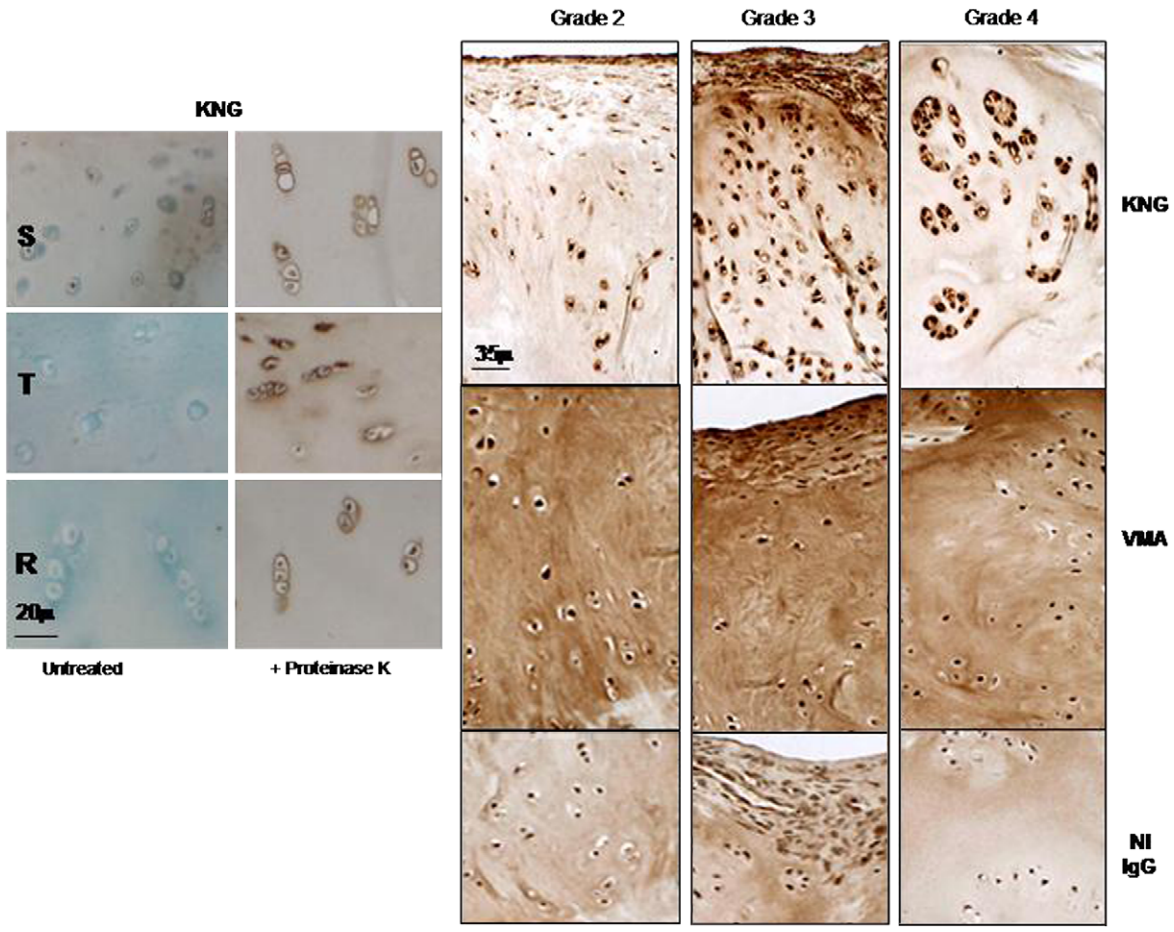

Fig. 4. Immunostaining with DAB of normal and OA cartilages for ADAMTS4 and 5. Normal cartilages from a 41-year female (left panels) taken from superficial (S), transitional (T) and radial (R) zones were stained with KNG with or without proteinase K treatment for antigen unmasking. OA cartilages (right panels, Grades 2, 3 and 4 from Patient 2) were proteinase K treated and stained with 10 μg/ml of nonimmune IgG, VMA (ADAMTS4) or KNG (ADAMTS5).

To further examine the organization of the enhanced staining of ADAMTS5 in OA tissues, KNG staining was next examined by confocal imaging (Fig. 5). As expected from the DAB data (Fig. 4) the cell-associated staining of ADAMTS5 was dramatically increased in OA relative to normal cartilage (left hand images, Figs. 5–7). Most strikingly, co-localization analyses with HABP showed abundant association of ADAMTS5 with HA surrounding cells in the superficial region of normal cartilage and in both the superficial and transitional regions of OA cartilages (indicated by white arrows, Figs. 5 and 6).

High magnification images of the cell-associated staining of the protease and HA (Figs. 6 and 7) revealed additional details of the spatial organization of the complexes. In the superficial zone of normal, and in both zones of OA cartilage, the immunoreactive enzyme was predominantly extracellular and appeared as a ''coat''-like structure, 1–3 microns thick, surrounding much of the cell. These HA–ADAMTS5 complexes, however, were not uniformly distributed around cells, but were seen as patchy foci localized along the periphery of individual cells in clonal groups (Figs. 6 and 7, white arrows). Notably, ADAMTS5 staining of cells in the transitional (Fig. 6) and radial (data not shown) zones of the normal cartilage was minimal; such immunoreactive enzyme was largely intracellular and no detectable co-localization with HA was seen (Fig. 6). To illustrate how the data in Figs. 6 and 7 are representative of the whole patient group under study we have provided further co-localization analyses of ADAMTS5 and HA in both normal and OA patients (Supplementary Figs. 6 and 7).

Co-localization analyses of ADAMTS4 (Fig. 8) and ADAMTS9 (Fig. 9) with HA provided no evidence for any complexes between these two proteases and HA. ADAMTS4 was not detected in normal cartilage, but was detectable as diffuse extracellular matrix staining at the surface of the OA cartilage, in general agreement with the DAB data shown in Fig. 4. ADAMTS9 staining (abundance and distribution) was similar in all zones of normal and OA cartilages (Fig. 9), showing a predominant intracellular or cell-associated filamentous distribution pattern. Interestingly, the compact cell-associated organization of this ''network'' in normal chondrocytes appeared to be modified in most of the OA cells in which there was a frequent occurrence of ADAMTS9-positive extensions protruding from the cell into the matrix (Fig. 9, white arrows).

WESTERN ANALYSIS OF ADAMTS PROTEINASES IN OA CARTILAGE

In order to examine the nature of the molecular forms of ADAMTS4, 5, 9 and 15 in human OA cartilages we carried out Western analysis on five separate preparations of

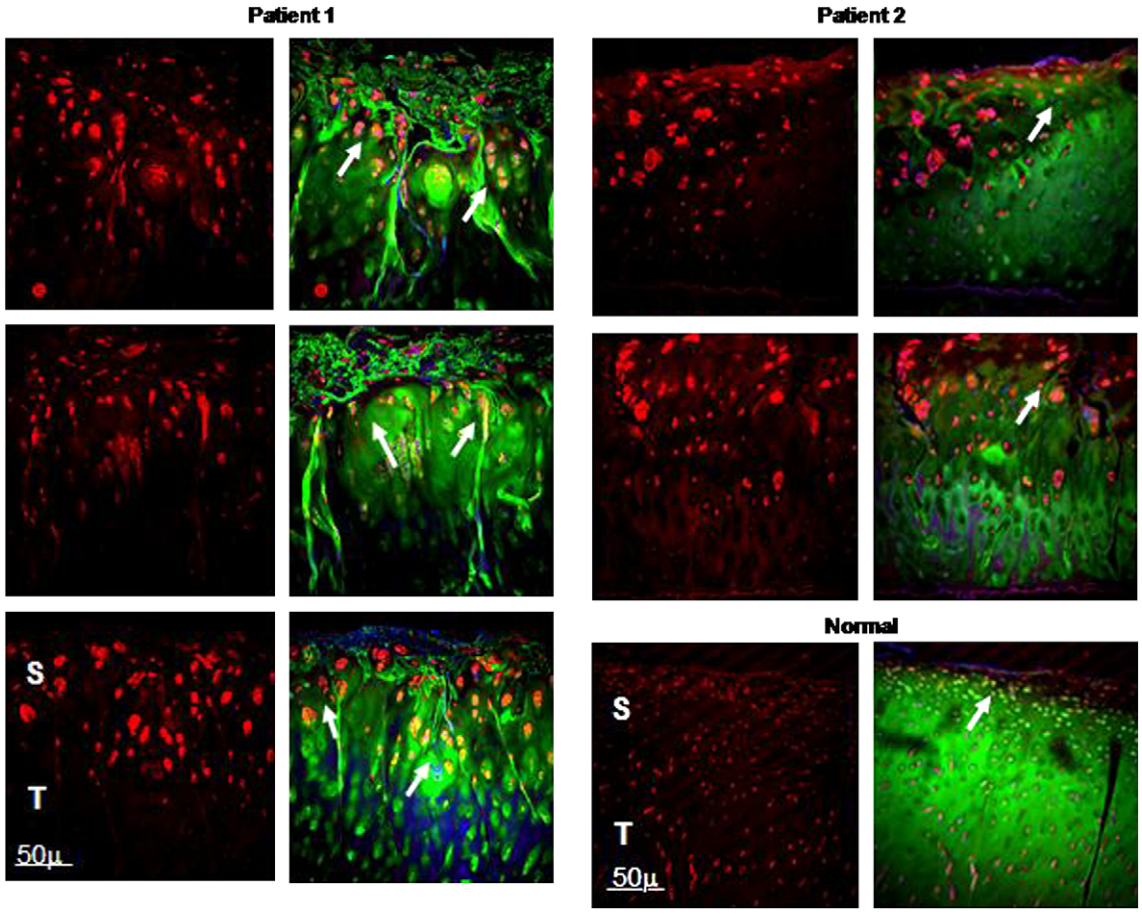

Fig. 5. Confocal imaging of normal and OA human cartilage co-stained for ADAMTS5 and HA. Cartilage sections (Grade 3.5) from OA Patient 1, OA Patient 2 and a normal 41-year female are shown. Each section was stained with KNG for ADAMTS5 (red), the HABP probe for HA (green) and TOTO-3 for nuclei (blue). Superficial (S) and transitional (T) regions of the sections were imaged by confocal microscopy. The left hand images show the protein stain only, and the right-hand images show the automated overlays (red + green + blue fluorescence) generated for each section. Areas appearing as yellow/orange indicative of GAG–protein co-localization are highlighted by the white arrows in each panel. Selected cell groups from such areas were examined at higher magnification and are shown in Figs. 6 and 7.

extracts of pooled OA tibial cartilages with and without digestion with *Streptomyces* hyaluronidase (see Methods for details). Crude extracts run under reducing conditions showed that ADAMTS4 was present as the expected p68 and p53 forms previously described[28], and this pattern was not altered by hyaluronidase digestion. ADAMTS9 was detected as a single species (about 60 kDa) and ADAMTS15 as two species (about 60 and 68 kDa) and neither pattern was altered by hyaluronidase digestion (data not shown).

A detailed Western analysis of ADAMTS5 protein in these extracts was undertaken next. This showed that digestion with *Streptomyces* hyaluronidase markedly increased the abundance of ADAMTS5 protein entering the gel, and also that the detection of ADAMTS5 with antibody KNG was markedly improved by electrophoresis under native conditions (without DTT reduction). To investigate the nature of the *Streptomyces* hyaluronidase-sensitive material, crude undigested extract was fractionated on Superose 6 and analyzed by Western blot for ADAMTS5 without reduction. The yield of high molecular weight complex (K_{av} 0–0.3) was low (see below for high yield procedure) and the majority of the recovered ADAMTS5 was eluted from Superose 6 with a K_{av} of about 0.6 and appeared as a 150 kDa complex on SDS-PAGE (for details see Discussion). In order to improve the recovery of the HA–ADAMTS5 complex from Superose 6, crude extracts were reduced and alkylated prior to chromatography to eliminate nonspecific protein–protein and protein–GAG interactions. Following this pretreatment, a low yield of 60 and 30 kDa ADAMTS5 species were found to be eluted from Superose 6, peaking in fractions 7–9 (Fig. 10, left panel), with a trailing edge of the same composition in fractions 10–12 (not shown). Most significantly, when these peak fractions were digested with *Streptomyces* hyaluronidase before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 10, middle panel) there was a very marked increase in the abundance of ADAMTS5 protein at about 150 kDa (diffuse band), 60 kDa and particularly 40 kDa. These species were also seen on digestion with Chase ABC, with an even higher yield of the diffuse band above 150 kDa. These biochemical data are consistent with the interpretation that ADAMTS5 is present in human OA cartilage extracts in multiple forms, all of which are sequestered in a HA-based complex. Furthermore, such a complex can be readily isolated for study by isotonic salt extraction and rapid chromatographic separation.

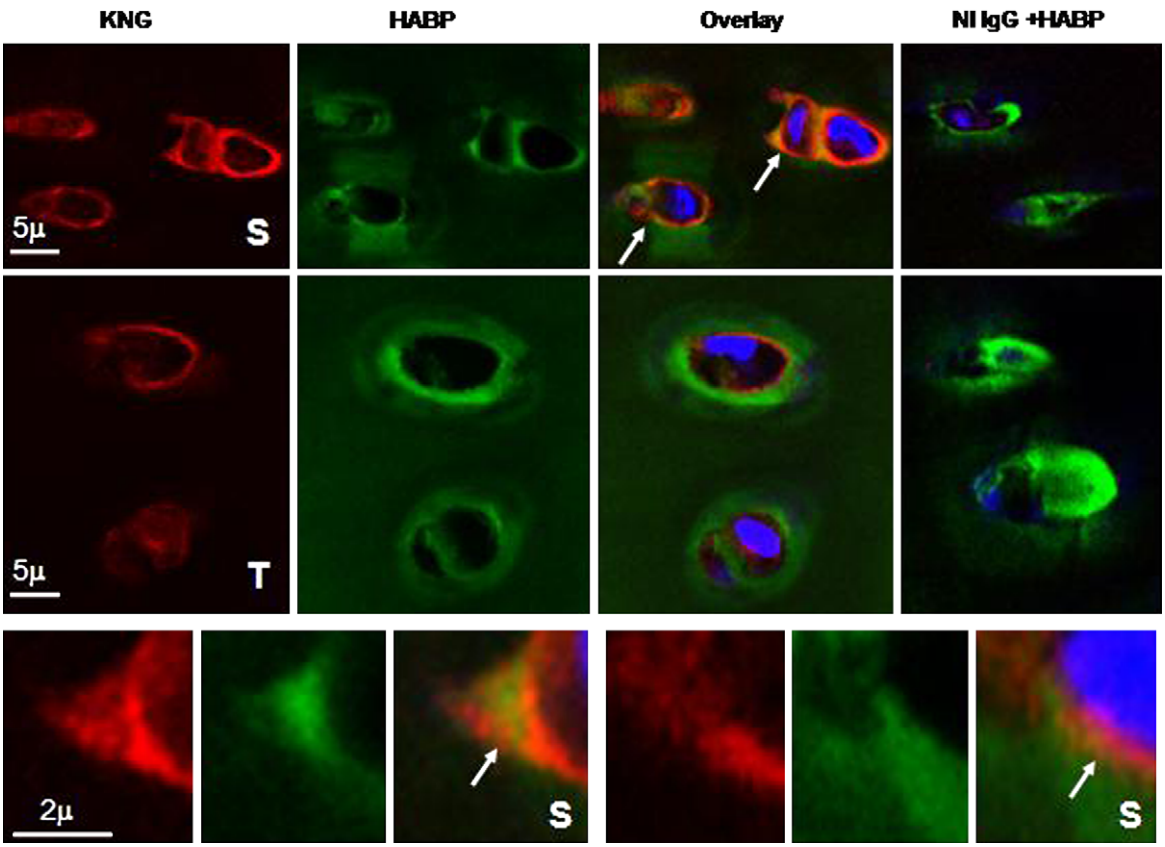

Fig. 6. High resolution ADAMTS5–HA co-localization in chondrocytes in normal cartilage. Representative cell groups from the superficial (S), and transitional (T) zones of normal cartilage sections stained with KNG (or nonimmune IgG) and co-stained with HABP were viewed at higher magnification (63×). KNG alone (red), HA alone (green) and overlays of the same cell group with KNG (red), HA (green) and nuclei (blue) are shown. Regions of co-localization of ADAMTS5 and HA (yellow–orange, bottom panels) are indicated by white arrows. See text for more details.

Discussion

Because of the widespread use of total knee replacement surgery, tissues from advanced disease are widely available for analysis. Many studies, including the present report, have focused on the fissured and remodeled articular cartilage, which remains on the tibial surfaces. Histologic description of this tissue[9] reports surface roughening, vertical fissures, surface delamination, cavity formation, tissue fragmentation and growth of fibrocartilage. Biochemical analysis has also revealed major changes in matrix composition and cellular activity at both the anabolic[48] and catabolic[49] levels. Cellular changes are primarily described as development of clonal nests, although there is considerable debate over whether these cells are derived from the original "quiescent" articular chondrocyte population by trans-differentiation[20] or whether they are migratory cells which enter from surrounding tissues[50,51].

While the mechanism by which these cells mediate the chronic and irreversible ECM destruction in OA remains unclear, there appears to be a general consensus that they represent the "osteoarthritic" cell, which is responsible for the progressive deterioration of the tissue. In this regard there is also consensus that ADAMTSs (such as ADAMTS4/aggrecanase-1 or ADAMTS5/aggrecanase-2) are responsible for destruction of the aggrecan matrix[22] whereas MMPs (such as MT1-MMP and MMP-13) destroy the collagen matrix[52]. On the other hand, there is no detailed information on temporal and spatial aspects of metalloproteinase abundance in OA development and little is known about how the different proteinase cascades may interact in space and time, to accelerate or limit matrix destruction. We report here data obtained from confocal immunohistochemical imaging of thin sections of full-depth human knee cartilage tissue, and in combination with established biochemical studies, this approach represents a new framework for studies of this area.

In the present study, ADAMTS5 (but not ADAMTS1, 4, 8, 9, and 15) was found to co-localize with HA around isolated cell groups in the superficial zone of normal adult cartilage and most strikingly around cells of clonal nests in OA cartilage. This co-localization *in situ* was reflected in the presence of an ADAMTS5–HA complex in tissue extracts (Fig. 10). This complex was isolated using Superose 6 chromatography of reduced and alkylated cartilage extracts, eluting with a $K_{av} \sim 0.3$, consistent with a molecular size of about 2×10^6. Stability of the complex to reduction and alkylation suggests that the capacity of ADAMTS5 to bind HA does not depend on globular protein structures maintained by disulfide bonding, but rather short linear peptide sequence such as the double HA-binding motif (Fig. 10). The purified complex was found to contain ADAMTS5 in multiple molecular forms (150, 60, 40 kDa), all of which were released by digestion with *Streptomyces*

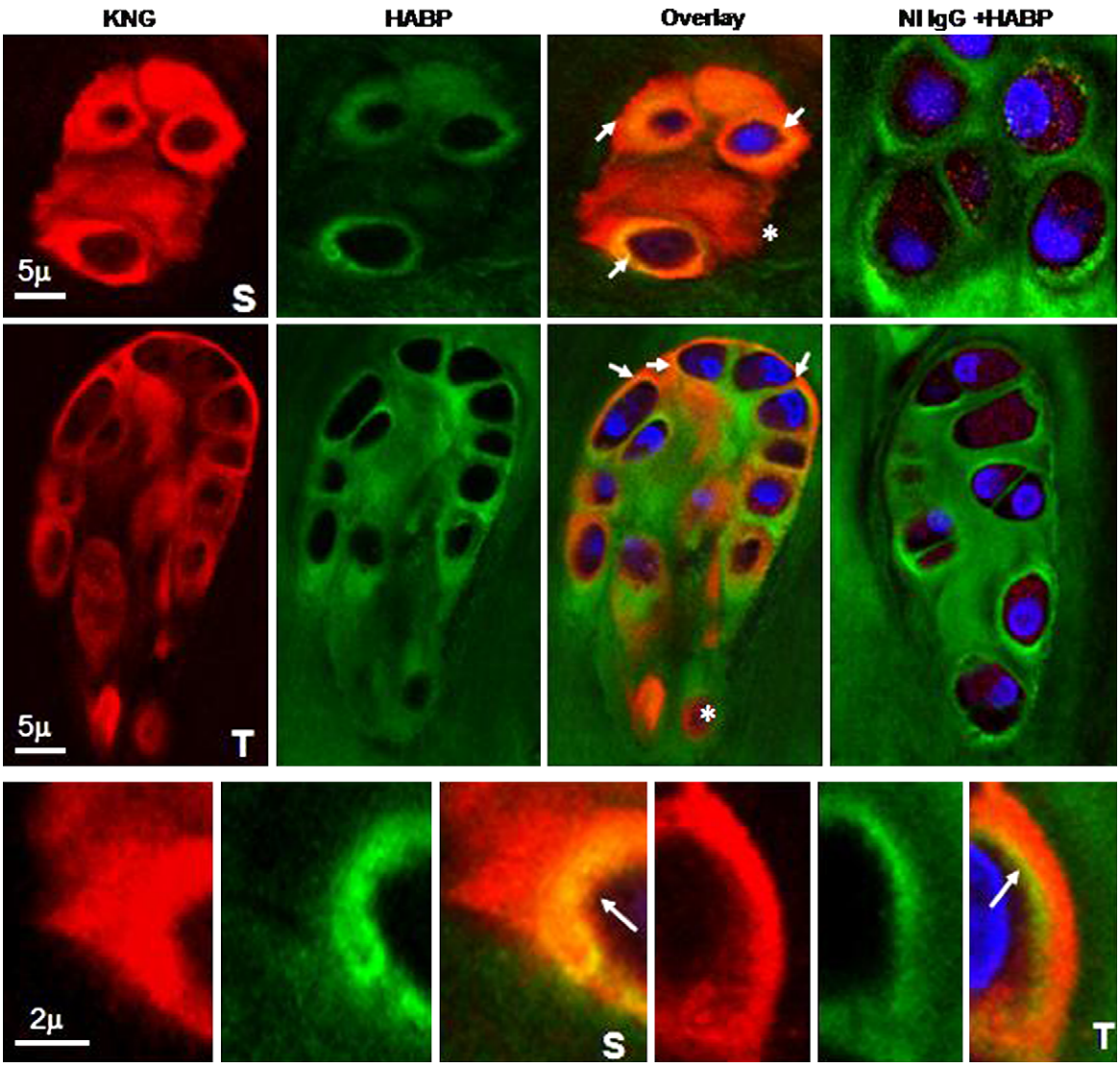

Fig. 7. High resolution ADAMTS5–HA co-localization in chondrocytes in the superficial zone of OA cartilage (Patient 1). Representative cell groups from the superficial (S) and transitional (T) zones' sections of OA cartilage (Patient 1) stained with KNG (or nonimmune IgG) and co-stained with HABP were viewed at higher magnification (63×). KNG alone (red), HABP alone (green) and overlays of the same cell group with KNG (red), HA (green) and nuclei (blue) are shown. Regions of co-localization of ADAMTS5 and HA (yellow–orange, bottom panels) are indicated by white arrows. HA-free areas' staining for KNG are indicted by (*). See text for more details.

hyaluronidase or chondroitinase ABC. A 55 kDa ADAMTS5 species has previously been characterized by us employing IHC and Western analysis of murine cumulus oocyte complexes with antibody KNG[37].

The major ADAMTS5 species released by HA digestion was a 40 kDa form which would appear to represent the fully activated form of the proteinase[53], processed by pro-domain removal and C-terminal truncation to remove the TSR2, spacer domain and much of the Cys-rich domain (see Fig. 10 diagram). The precise C-terminal of this species is unknown, however, by comparison with SDS-PAGE migration of recombinant forms[53] it would appear to be near the boundary of the TSR1 and Cys-rich domains. The finding that it is detected by antibody KNG (residues 636–650) shows that the protein must include these residues and it therefore appears to represent the ADAMTS5 equivalent of the highly active p40 form of ADAMTS4 which is generated by autocatalytic and MT4-MMP-mediated removal of the spacer and Cys-rich domains[26]. The diffusely migrating ADAMTS5-positive product of about 150–200 kDa might retain undigested HA oligosaccharide which might be explained if the binding proteins prevent complete HA cleavage. In this regard it is interesting that, in addition to ADAMTS5, this species contains a 65 kDa form of heavy chain 2[54] (Plaas and Yoshihara, unpublished), a finding which appears to be related to the presence of SHAP (heavy chain–HA) complexes in the synovial fluid of RA patients[55].

The discovery here of a complex of HA and ADAMTS5 (but not ADAMTS1, 4, 8, 9, 15) is consistent with the GAG-binding motifs present within the C-terminally truncated forms of these proteinases (see Fig. 10). On the basis that a BX7B motif confers HA binding[56] whereas an XBBXBX motif confers heparin binding[57], there are four potential binding sites present in the aggrecanases, two contiguous HA-binding motifs within the disintegrin domain, and two separated

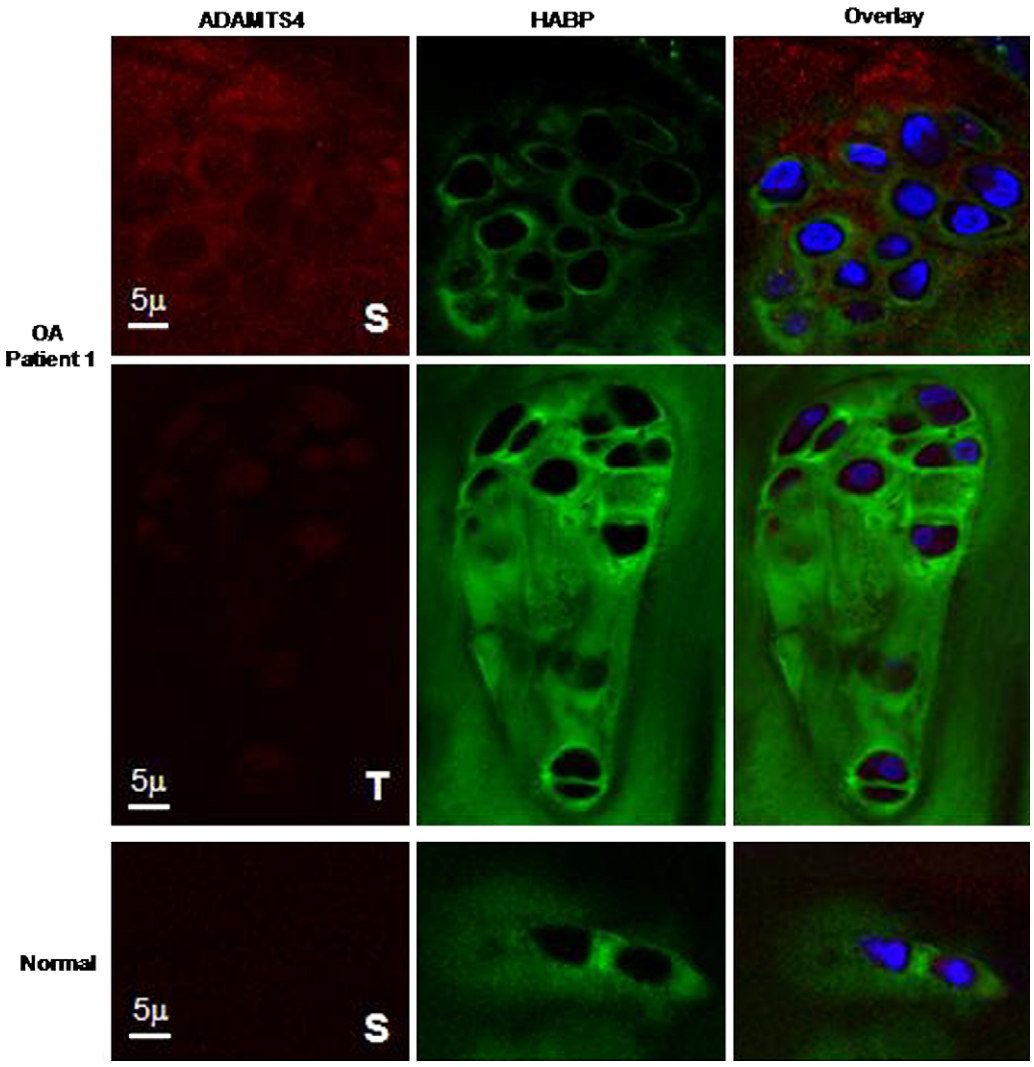

Fig. 8. ADAMTS4–HA co-localization in chondrocytes in the superficial zone of normal and OA cartilage. The localization of ADAMTS4 in relation to HA was examined in cell groups from the superficial (S) and transitional (T) zones in sections of normal and OA cartilages. VMA alone (red), HABP alone (green) and overlays of the same cell group with VMA (red), HA (green) and nuclei (blue) are shown.

heparin-binding motifs within the thrombospondin and spacer domains. ADAMTS5 is unique in containing both HA-binding motifs (544KGRICLQGKCVDKTKKK559) within the disintegrin domain, consistent with a high affinity for HA of all molecular forms including the activated p40 form which is so abundant in the isolated complexes. It is also worth mention that human TIMP-3, a potent inhibitor of the aggrecanases[58] has three BX7B HA-binding motifs.

It should also be noted that the ADAMTS5–HA complexes were confined to specific regions of the pericellular HA coat (Figs. 6 and 7), and this is consistent with a regulated assembly of this complex. This may occur in association with cellular dynamics, such as proliferation, migration and cell–cell or cell–matrix adhesion in the formation of clonal nests. In this regard it may be relevant that specifically structured HA matrices are generated at many pericellular locations involving response to stresses such as viral[59], oxidative[60] and inflammatory insult[61]. The formation of these HA complexes requires TSG-6[62], which is also found in high abundance by immunostaining within the clonal nests of OA cartilages (Plaas, Osborn and Sandy, unpublished). In this regard, the TSG-6-mediated formation of an HA complex capable of sequestering ADAMTS5 protein and HC2 may explain the chondroprotective effect of TSG-6 overexpression in murine inflammatory arthritis[63].

The formation of such proteinase–HA complexes in human cartilage could also be protective against aggrecanase-mediated tissue destruction. This would be analogous to the role played by HA in protecting the integrity of the pericellular environment of airway epithelium by sequestering kallikrein and lactoperoxidase, whose rapid actions at select sites are nonetheless necessary to maintain tissue homeostasis[64]. On the other hand, it is possible that persistent and/or excessive accumulation of such proteinase–HA complexes in clonal nest in OA cartilage, followed by uncontrolled dissolution of the HA through hyaluronidase activity[65] or oxyradical cleavage[66] releases the p40 form of ADAMTS5 and generates the pathological activity that results in progression of murine OA[67] and also possibly human disease.

Clearly, more work is needed to determine whether the co-localization of ADAMTS5, but not ADAMTS1, 4, 8, 9 and 15, with HA in human OA cartilage represents evidence that ADAMTS5 is the destructive aggrecanase in this tissue. Our antibody design and characterization suggests

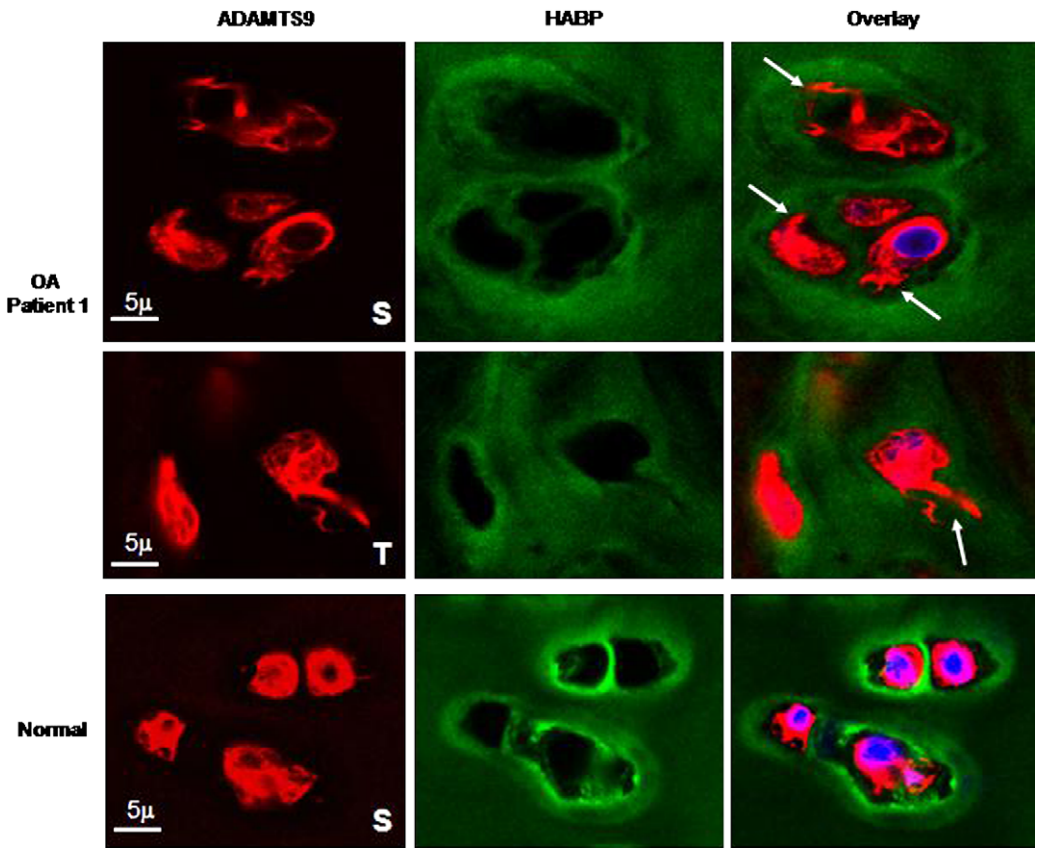

Fig. 9. ADAMTS9–HA co-localization in chondrocytes in the superficial zone of normal and OA cartilage. The localization of ADAMTS9 in relation to HA was examined in cell groups from the superficial (S) and transitional (T) zones in sections of normal and OA cartilages. FDG alone (red), HABP alone (green) and overlays of the same cell group with FDG (red), HA (green) and nuclei (blue) are shown.

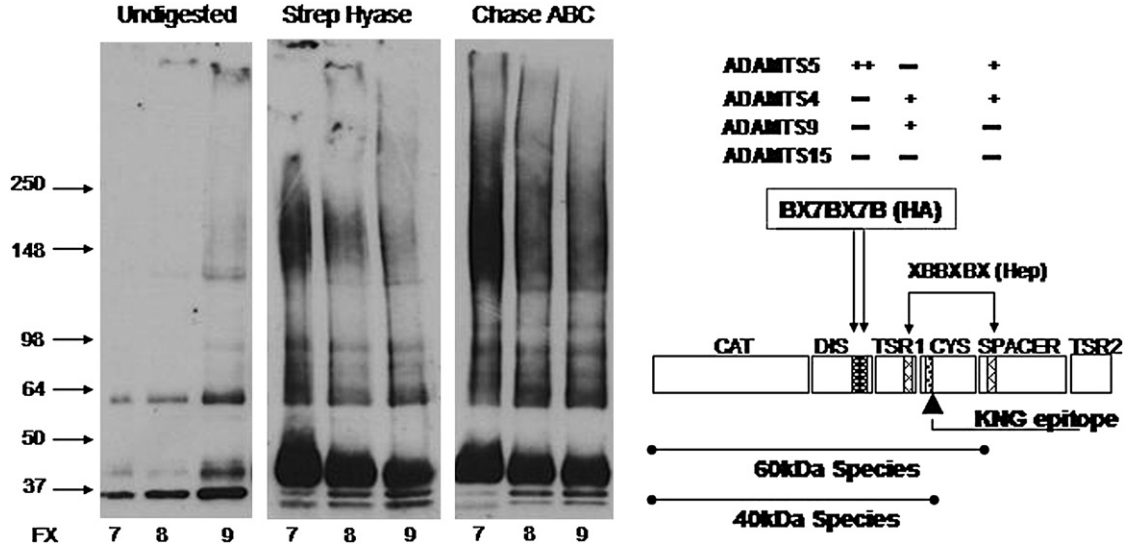

Fig. 10. Western analysis of the HA–ADAMTS5 complex isolated from OA cartilage and the location of GAG-binding motifs in the different enzymes. Isotonic salt extract of human OA cartilages was reduced and alkylated for Superose 6 chromatography. Fractions were characterized by Western analysis under nonreducing conditions with antibody KNG. Only data from peak fractions 7–9 are shown. Samples were loaded undigested or after digestion with *Streptomyces* hyaluronidase or Chase ABC. The schematic shows the location and presence (+) or absence (–) of HA-binding (BX7B) and heparin-binding (XBBXBX) motifs in human ADAMTS4, 5, 9 and 15. Also shown are the location of the KNG epitope for ADAMTS5 and the predicted structures of the 60 and 40 kDa forms of ADAMTS5. See text for more details.

that the lack of staining for ADAMTS1 and ADAMTS8 is not due to poor quality antibodies but due to the low abundance of these aggrecanases in the human cartilage specimens studied here. In this regard, it should be noted that the antibodies to ADAMTS1 (HNE), ADAMTS5 (KNG), ADAMTS8 (YNA), ADAMTS9 (FDG) and ADAMTS15 (HST) were all raised against ''equivalent'' peptides located at the same site at the N-terminal end of the Cys-rich domain of the respective proteins. This region shows very little sequence similarity between these five aggrecanases and so peptides at this location can be used to generate member-specific antibodies. We also have an anti-ADAMTS4 antibody to this region (YNH)[28], however, the ADAMTS4 antibody (VMA)[28] used in the present study was raised against a sequence in the catalytic domain. While the relative reactivities of these anti-ADAMTS IgGs for their cognate epitopes have not been determined, all antibodies readily detected the recombinant proteins on Western analysis which were loaded on gels at equivalent volumes (50 μl) of conditioned medium from the transfected cells.

With respect to which aggrecanase is most important in the pathology of human OA, Wachsmuth *et al.*[68] have examined ADAMTS1 abundance in normal and OA human cartilages and have concluded that ADAMTS1 is not responsible for pathological aggrecanolysis. Malfait *et al.*[69] studied ADAMTS enzyme abundance and the effects of ADAMTS inhibitors on aggrecanolysis in human cartilage explants and concluded that both ADAMTS4 and ADAMTS5 are responsible. Demircan *et al.*[70] compared the IL-1-inducibility of ADAMTS1, 4, 5, 8, 9 and 15 in human chondrocytes and showed that ADAMTS9 is most readily induced and therefore may be responsible for human cartilage pathology. An important question for future study in this area is the identification of biologically relevant substrates for the aggrecanases. While most work has focused on aggrecan degradation in cartilage it is also now clear that these proteinases have activity *in vivo* against other substrates in joint tissues such as versican V0/V1[41], COMP[71] and alpha-2-macroglobulin[72]. The physiologic importance of these activities, the effect of C-terminal truncation on the activity and specificity of ADAMTS4 and ADAMTS5 and the control of ADAMTS4 and ADAMTS9 activity by furin-mediated pro-domain removal[73,74] also clearly represent important areas of investigation. On the other hand, the present study further suggests that an understanding of the role of HA in cartilage aggrecanolysis[75] may provide a definitive clue to the means of effective therapeutic control of joint tissue destruction.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.joca.2006.12.008.

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