HHS Public Access

Author manuscript

Matrix Biol. Author manuscript; available in PMC 2016 October 01.

Published in final edited form as:

Matrix Biol. 2015 October ; 48: 42–54. doi:10.1016/j.matbio.2015.04.002.

CD44 knock-down in bovine and human chondrocytes results in release of bound HYAL2

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Abstract

CD44 shedding occurs in osteoarthritic chondrocytes. Previous work of others has suggested that the hyaluronidase isoform HYAL2 has the capacity to bind to CD44, a binding that may itself induce CD44 cleavage. Experiments were developed to elucidate whether chondrocyte HYAL2: (1) was exposed on the extracellular plasma membrane of chondrocytes, (2) bound to CD44, (3) underwent shedding together with CD44 and lastly, (4) exhibited hyaluronidase activity within a near-neutral pH range. Enhancing CD44 shedding by IL-1β resulted in a proportional increase in HYAL2 released from human and bovine chondrocytes into the medium. CD44 knockdown by siRNA also resulted in increased accumulation of HYAL2 in the media of chondrocytes. By hyaluronan zymography only activity at pH 3.7 was observed and this activity was reduced by pre-treatment of chondrocytes with trypsin. CD44 and HYAL2 were found to co-immunoprecipitate, and to co-localize within intracellular vesicles and at the plasma membrane. Degradation of hyaluronan was visualized by agarose gel electrophoresis. With this approach, hyaluronidase activity could be observed at pH 4.8 under assay conditions in which CD44 and HYAL2 binding remained intact; additionally, weak hyaluronidase activity could be observed at pH 6.8 under these conditions. This study suggests that CD44 and HYAL2 are bound at the surface of chondrocytes. The release of HYAL2 when CD44 is shed could provide a mechanism for weak hyaluronidase activity to occur within the more distant extracellular matrix of cartilage.

Keywords

CD44; hyaluronan; hyaluronidase; chondrocyte

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Ethical statement

The authors have nothing to declare.

1. Introduction

The loss of aggrecan from articular cartilage is an early critical event associated with osteoarthritis (OA) [1]. Enhanced aggrecan turnover within the extracellular matrix occurs due to the enhanced activity of endoproteinases such as ADAMTS4 [2, 3] and ADAMTS5 [4–6] as well as other matrix metalloproteinases (MMPs) [7–10]. In addition to aggrecan, a significant loss of hyaluronan (HA) is also observed in human OA cartilage as compared to normal human cartilage [11–14]. In our studies, cultured explants of human articular cartilage treated with IL-1α displayed a loss of HA within the superficial and upper middle layers of cartilage—the same layers in which aggrecan loss also occurred [14]. Although HA is clearly lost, the mechanism for HA turnover remains unclear. In chondrocytes the HA receptor, CD44 mediates the endocytosis of cell-associated HA and HA-retained aggrecan fragments [15, 16]. Most of the internalized HA is destined for complete catabolism intracellularly via the action of lysosomal hyaluronidases, β-glucuronidases and hexosaminidases. The hyaluronidases are represented by a family of β-endoglucosidases that degrade HA by cleaving internal β-1, 4 linkages [17–19]. Six hyaluronidase genes have been identified in humans [19]. Cartilage has been shown to express mRNA encoding HYAL1, HYAL2 and HYAL3 and of these three hyaluronidases, hyaluronidase 2 (HYAL2) is the predominant mRNA transcript expressed by bovine and human chondrocytes [20, 21]. Although CD44-mediated endocytosis likely accounts for a large portion of the cell-associated and perhaps pericellular HA turnover, questions have long been raised whether there is hyaluronidase activity within the more distant extracellular matrix [22].

HYAL2 has been of considerable interest as a candidate extracellular hyaluronidase. In some cell types, HYAL2 exists as a glycosylphosphatidylinositol-linked (GPI-linked) membrane protein [23, 24] exposed to the extracellular milieu. Additionally, others have also shown that the HYAL2 hydrolase binds to CD44 and is co-internalized with CD44 and HA into endosomes [25, 26]. Nonetheless debate continues on the subcellular localization of HYAL2, and more importantly, whether the enzyme exhibits any HA-degrading activity within extracellular environments wherein the pH is likely considerably higher than that of late endosomes or lysosomes [23–27]. In our previous work we could only detect hyaluronidase activity in HYAL2-transfected chick embryonic chondrocytes at pH 3.7 conditions [20]. Later, Harada and Takahashi demonstrated HYAL2 hyaluronidase activity within the pH range of 6.0 – 7.0, in stably-transfected HEK293 cells but, the activity was dependent on the expression of CD44 [28]. Neutral pH hyaluronidase activity also required that the assays be performed using intact cells or non-detergent-solubilized membrane fragments. One conclusion that could be drawn from this was that neutral pH HYAL2 activity required a continued association with CD44.

When HYAL2 was overexpressed in a rat fibroblast cell line, enhanced shedding of CD44 from the cell surface was observed [26]. This shedding was similar to treating control fibroblasts with HA oligosaccharides. This was of interest because we have recently observed substantial CD44 fragmentation associated with human OA chondrocytes [29]. CD44 cleavage could also be induced in bovine chondrocytes via progressive passage in culture (de-differentiation), treatment with HA oligosaccharides or exposure to IL-1β [29]. The cleavage of CD44 in chondrocytes involves a sequential two step mechanism including

the action of a membrane-bound metalloproteinase (the “sheddase”) followed by intra-membranous cleavage by a γ-secretase. Taken together, these results suggest a connection between HYAL2 localization and CD44 shedding.

In this study we set out to investigate whether there is a linkage between extracellularly-localized HYAL2, CD44 and the enhanced shedding of CD44 that occurs in association with OA. Another question addressed was whether an association of CD44 and HYAL2 proteins does in fact affect the pH dependent hyaluronidase activity in chondrocytes.

2. Results

2.1. Anti-human HYAL2 antibody detects a 54 kD band in human OA chondrocytes

Human OA chondrocytes derived from the knee cartilage of several patients were grown as high-density monolayers for 2 days and total cell lysates collected. Western blots developed using the anti-human HYAL2 antibody demonstrated a predominant single band for HYAL2 at ~54 kD (Fig. 1A, arrow) although numerous, likely non-specific, additional bands were also present. Western blots developed using the anti-CD44 cytotail antibody demonstrated a broad band typical for full-length CD44 centered at 85 kD as well as numerous low molecular mass bands for CD44 (Fig. 1B, lanes labeled Ctr). We have previously demonstrated that these bands are indicative of the sequential proteolytic cleavage and shedding of CD44 from the cell surface as part of a two-step process [29]. Cleavage of the extracellular domain of CD44 by an MMP or ADAM results in C-terminal products 20–25 kD that remain within the membrane (CD44-EXTs, Fig. 1B boxed area). The CD44 EXT fragments are then cleaved within the intramembranous domain by a δ-secretase, releasing a 15 kD small intracellular domain (CD44-ICD) into the cytoplasm. The baseline levels of CD44 EXT and ICD fragments vary widely from patient to patient but, the accumulation of these fragments can be reduced by incubation of the chondrocytes with the MMP inhibitor GM6001, ranging from 22% to 44% decrease, following relative quantification of the scanned CD44 EXT bands as compared to untreated controls (Fig. 1B, lanes labeled GM). Western blot analysis of direct human OA cartilage extracts also revealed full length CD44 (Fig. 1C), CD44 fragmentation (Fig. 1C, boxed area) as well as HYAL2 (Fig. 1C, arrow). CD44 fragmentation can be enhanced, even in OA chondrocytes, by exposure to IL-1β; western blots demonstrated the bands (Fig. 1D, boxed area; EXTs) and relative quantification of these bands reflect the fold-stimulation of CD44-EXTs (Fig. 1D, bar graph). In the same cell lysates, the 54 kD band indicative of HYAL2, was present but displayed no change in intensity with IL-1β treatment (Fig. 1E, arrow). However, HYAL2 present in the culture media of the same cell cultures displayed enhanced accumulation following treatment with IL-1β (Fig. 1E); relative quantification of these HYAL2 bands reflect this fold-increase (Fig. 1E, bar graph). Interestingly, only the 54 kD band was observed in the medium fraction.

2.2. Correlation of HYAL2 release with CD44 shedding

The results in Figure 1 suggested that shedding or release of HYAL2 might be associated with the shedding of CD44. To investigate this possibility, bovine chondrocytes were treated with or without GM6001. As shown in Figure 2, GM6001 diminished the generation of

CD44 EXT bands detected in cell lysates (Fig. 2A, boxed area) which was coincident with a reduction in HYAL2 released into the medium (Fig. 2B, asterisks). Note that bovine articular chondrocytes responded in a fashion similar to human OA chondrocytes. The bovine chondrocytes exhibit enhanced CD44 fragmentation in response to 1 and 5 ng/ml IL-1β—degradation that can be blocked by GM6001 and, differential accumulation of a single 54 kD HYAL2 in the culture medium. As CD44 fragmentation was decreased by treatment with GM6001, accumulation of HYAL2 in the media also decreased (Fig. 2C). Therefore, with decreased CD44 fragmentation, there was a decrease in HYAL2 found in the media of these bovine chondrocytes even in the presence of IL-1β.

2.3. Validation of the 54 kD protein band as HYAL2 protein and correlation with hyaluronidase activity

Several approaches were used to validate that the 54 kD protein band observed by western blotting in Figures 1 and 2 represented HYAL2. Hyaluronidase activity was measured by HA zymography at pH 3.7. Cell lysates from bovine chondrocytes exhibited two bands indicative of hyaluronidase activity when assayed under low pH 3.7 conditions (Fig. 3A), migrating as proteins of approximately 40 and 54 kD. Medium fractions from these same cultures exhibited a chief single band with hyaluronidase activity at ~54 kD; the intensity of that band increased following treatment with IL-1β (Fig. 3A). Relative quantification of these HYAL2 bands reflects this fold-increase in the hyaluronidase activity in the media following treatment with IL-1β (Fig. 3A, bar graph). In a loss-of-function approach, bovine chondrocytes were transfected with control-siRNA (labeled C) or HYAL2-specific siRNA (labeled siRNA). As shown in Fig. 3B, HYAL2-specific siRNA knockdown resulted in a diminution of the larger, 54 kD band when visualized by western blotting (WB) developed with an anti-HYAL2 antibody. This reduction by siRNA was paralleled by a reduction in intensity of hyaluronidase activity of the upper bands observed in HA zymograms at 48 hours post-transfection (Fig. 3B). Relative quantification of the WB and the HA zymogram bands reflects those decreases (Fig. 3B, bar graph). As a gain-of-function approach, bovine chondrocytes were transfected with an empty-vector (labeled C) or, a pSPORT-HYAL2 expression plasmid (pHYAL2). Twenty-four and 48 hours after transfection, cell lysates and media were collected and equivalent protein aliquots analyzed by HA zymography. As shown in Fig. 3C, two hyaluronidase activity bands were detected at pH 3.7 in the range of 40 to 54 kD in lysates from cells transfected with an empty expression plasmid, likely depicting the endogenous hyaluronidase as in Fig. 3A. The corresponding media fractions exhibited the 54 kD hyaluronidase activity band. Following HYAL2-plasmid transfection, there was a prominent increase in intensity of the upper band (~54 kD) in both the cell lysates and the media fraction (Fig. 3C). The increase in the cell lysate hyaluronidase activity seen at 24 hours was decreased by 48 hours; concomitantly, the increase in the media was larger at 48 hours than at 24 hours, possibly indicative of shedding from the cell surface during this time frame. Thus, these three sets of data strongly suggest that the upper ~54 kD band represents HYAL2 and moreover exhibits hyaluronidase activity at pH 3.7.

Interestingly, when pHYAL2-transfected chondrocytes were treated with trypsin prior to detergent lysis, the intensity of the 54 kD band was eliminated leaving a single lower band with residual activity (Fig. 3D). This indicates that the 54 kD activity was localized

extracellularly and sensitive to trypsinization. Nonetheless, HA clearing activity in these HA zymographs was only detected under pH 3.7 incubation conditions (Fig. 3E). No cellular hyaluronidase activity was observed at higher pH conditions such as pH 5.0 wherein the activity of the positive control namely, commercial testicular hyaluronidase, is clearly active at pH 5.0 (Fig. 3E) [20]. Thus the 54 kD band (in Fig. 3D) represents hyaluronidase with activity at a low pH.

2.4. siRNA knockdown of CD44 results in increased HYAL2 in the culture media

To determine whether CD44 plays a role in HYAL2 retention, bovine (Fig. 4A, C) and human (Fig. 4B, D) chondrocytes were transfected with a CD44-specific or control siRNA. Western blots developed using an anti-CD44 cytotail antibody revealed a ~50% decrease knockdown of full length bovine and human CD44 in cells treated with CD44-siRNA (as compared with control-siRNA), at both 24 and 48 hours post-transfection. Under these same conditions, more HYAL2 accumulated in the medium in both bovine (Fig. 4C) and human (Fig. 4D) chondrocytes in association with the knockdown of CD44 at both 24 and 48 hours. In contrast, the 54 kD bands in the cell lysates displayed no apparent change in protein levels with CD44-siRNA (Fig. 4C, 4D). Thus, retention of HYAL2 appears to be dependent on the expression of CD44 (Fig. 4E).

2.5. HYAL2 co-immunoprecipitates with CD44

To determine whether CD44 and HYAL2 interact directly, co-immunoprecipitation experiments were performed using C28/I2 cells, an immortalized human chondrocyte cell line that exhibits less extracellular matrix which may be a confounding factor in this technique. First, immunostaining of C28/I2 cells revealed CD44 primarily localized to the plasma membrane, and sensitive to trypsin treatment for its removal (Fig. 5A). HYAL2 immunostaining tends to be peripherally-localized in these cells but with a punctuate appearance. Moreover, a large percentage of the immunostainable HYAL2 was released with trypsin (Fig. 5A) Similar to the HA zymograms shown in Fig. 3D, western blotting of lysates obtained before and after trypsinization demonstrated a significant decrease in the 54 kD bands for HYAL2 in C28/I2 cells following the trypsin-treatment, as well as in bovine chondrocytes used for comparison (Fig. 5B). These results show that C28/I2 cells are similar to human OA and bovine chondrocytes with respect to CD44 and HYAL2 expression and localization.

Following two experimental cell culture approaches, C28/I2 cell lysates were immunoprecipitated with an anti-CD44 antibody and probed for HYAL2 on western blots (Fig. 5C, D). In the control (Ctr) lanes in Fig.5C and 5D, a 54 kD band indicative of co-immunoprecipitating HYAL2 was clearly observed, including the hint of a slightly smaller doublet band. In the first experimental approach, CD44-specific siRNA was used to knockdown CD44 expression prior to preparing lyates (Fig. 5C). As compared to C28/I2 cells transfected with a control siRNA, 43% less HYAL2 co-immunoprecipitated with CD44 under CD44 knock-down conditions. In the second experimental approach, C28/I2 cells were trypsinized (+ Tryp) prior to preparing lysates. Again, as compared to untreated C28/I2 cells, 54% less HYAL2 co-immunoprecipitated with CD44 (Fig. 5D) although this is due in part to trypsin degradation of the CD44. Although the amount of HYAL2 present in the

immunoprecipitates in each of these two experiments differed, it is noteworthy that none of non-specific bands typically observed in total cell lysates, such as seen in Figure 1A, were present. These results indicate that HYAL2 does have the capacity to directly interact with CD44 and, of the multiple bands that might represent HYAL2 (or nonspecific bands) only the 54 kD protein associates with CD44.

2.6. Functional significance of CD44 / HYAL2 interactions

Although the retention of HYAL2 to the chondrocyte cell surface via CD44 is evident, the significance of this interaction is unknown. It is unclear what role extracellular HYAL2 might play if there is little enzymatic activity of this enzyme, even at pH 5.0, as shown in Fig. 3E. However, reports by Harada and Takahashi [28] demonstrated HYAL2-dependent hyaluronidase activity at more neutral pH (pH 6.8) when 1) HYAL2 and CD44 were over-expressed in HEK293 cells and 2) when assayed for activity as non-solubilized membrane fractions. Their results suggest that HYAL2 exhibits hyaluronidase activity at a neutral pH range but only when there is direct physical interaction between HYAL2 and CD44. To address whether such an activity occurs naturally in articular chondrocytes, hyaluronidase activity in non-transfected chondrocytes was examined. The degradation of FITC-conjugated HA in solution was used as the assay. Changes in HA size can be readily observed on 1% agarose gels. Fig. 6A illustrates the range in separation of commercially-available HA preparations differing in size from less than 5 kD to 1800 kD. A 15 second sonication of high molecular mass HA shears the HA to a more intermediate size. FITC-HA of high molecular mass HA before and after sonication can be readily visualized either by stains-all detection of the glycosaminoglycan (Fig. 6A) or transilluminator UV detection of the FITC-HA (Fig. 6B). When confluent cultures of C28/I2 cells were incubated with FITC-HA placed directly into the complete culture medium for 4 days, no change in the size of the medium HA was observed (Fig. 6C). In a next attempt to assure maximal CD44/HYAL2 interaction in the assay, CD44/HYAL2 co-immunoprecipitated complexes were generated from C28/I2 cell lysates. While still bound to the affinity resin, the immunoprecipitated complexes (similar to those shown in Fig. 5C, D) were incubated directly with high molecular mass FITC-HA at pH 6.8. After 24 or 48 hours of incubation at 37 °C, there was no significant change in mobility (size) of FITC-HA incubated with CD44/HYAL2 co-immunoprecipitated beads (lanes numbered as 3 in Fig. 6D) as compared to control beads with no lysates or antibodies bound (Fig. 6D, lanes 2) or, as compared to control FITC-HA that was not incubated with beads (Fig. 6D, lanes 1); although a slight but discernable shift to HA of lower size was observed at 48 hours. The small changes observed between the two controls, FITC-HA only or with control Dynabeads, represent non-enzymatic alterations of FITC-HA size, perhaps due to temperature or magnetic iron-induced free radical activity. After 72 or 96 hours of incubation, the change in FITC-HA size was more clearly evident, progressive and significant upon densitometric scanning of the migration of the fluorescent bands into the gel. These results support the suggestion that HYAL2, when bound directly to CD44, exhibits weak but observable hyaluronidase activity at a near neutral pH range. To determine whether this weak activity at pH 6.8 was a shoulder of hyaluronidase activity, optimum at a lower pH range of 4.5 to 5.5 as suggested by Bourguignon et al. [25], the assays were repeated under pH 4.8 conditions. As shown in Fig. 6E, degradation of the FITC-HA was clearly evident even at the 24 hour time point under these pH 4.8 conditions.

Thus, unlike the negative zymography results at pH 5.0 using detergent-solubilized cell lysates (Fig. 3G) hyaluronidase activity at pH 4.8 can be observed when HYAL2 is bound to CD44.

3. Discussion

The data from this study demonstrate that a close interaction exists between HYAL2 and CD44 in human and bovine chondrocytes. When CD44 cleavage is increased with IL-1β treatment, there is a coincident increase of HYAL2 that accumulates in the medium. This accumulation in the medium might be due to direct secretion and/or, shedding of HYAL2 from the cell surface. However, inhibition of CD44 shedding with GM6001 inhibits HYAL2 accumulation in the medium whereas siRNA mediated knock-down of CD44 enhances its release. While it is possible that manipulating CD44 may influence the synthesis and secretion of HYAL2, the most straightforward explanation is that CD44 is needed to retain HYAL2 at the cell surface. This was further validated by the demonstration that HYAL2 co-immunoprecipitates with CD44 and, evidence that a fraction of the cell surface-associated HYAL2, like CD44, is susceptible to trypsin. Interestingly Duterme et al. suggested that HYAL2 overexpression induces CD44 shedding [26]. Our work suggests that inducing CD44 shedding results in the release of HYAL2. This may be a chicken-egg conundrum with both mechanisms occurring.

Our results may represent an alternative mechanism for the retention of HYAL2 at the cell surface, namely retention via direct binding to CD44. In polarized epithelial cells, HYAL2 has been demonstrated to exist as a GPI-linked protein at the luminal surface [24–26]. In our previous work we could not detect the release of HYAL2 from chondrocytes using phosphatidylinositol-specific phospholipase C [20]. Nonetheless, GPI-HYAL2 is thought to localize in lipid rafts [25] and, we have shown that CD44 must transit into lipid rafts in order to undergo IL-1β-induced CD44 shedding [29]. It is also possible that CD44 localized in lipid rafts retains the extracellular HYAL2. Thus, our current results do not rule out the membrane retention of HYAL2 via both a GPI-linkage and binding to CD44, although the release of HYAL2 into medium would require a more complicated mechanism with GPI-linkages involved.

Gain-of-function (pHYAL2) and loss-of-function (HYAL2 siRNA) approaches highlight that HYAL2, in bovine chondrocytes, is functional as an active hyaluronidase at the lysosomal pH range. This differs from work in rat fibroblasts wherein overexpressed HYAL2 exhibited little activity in HA zymograms at pH 3.7 [26]. Given that considerable HYAL2 is localized intracellularly in chondrocytes (Fig. 5A), it is not unreasonable to suggest that HYAL2 functions as a lysosomal hyaluronidase. The question is whether HYAL2 exhibits hyaluronidase activity within a pH range anywhere near neutral that would indicate a functional relevance to its location in the extracellular environment. On HA zymograms, no activity was detected at pH 5.0 (Fig. 3F). However, the SDS-electrophoretic gel approach used for zymograms separates HYAL2 as an individual 54 kD protein and would dissociate any non-covalent interaction with CD44. Co-immunoprecipitation of HYAL2 with CD44 (Fig. 5C, 6E) served as a useful way to purify HYAL2 and moreover, only HYAL2 that was bound to CD44. We explored the use of whole membrane fractions

prepared from RCS chondrocytes, as described by others [28] but, although we could detect degradation of soluble FITC-HA or biotin-bound HA by ELISAs, the particulate-membranes likely interfered with these assays yielding inconsistent results. With the CD44/HYAL2/Protein G-bead complexes intact, a weak but clearly detectable FITC-HA degradation was observed at pH 6.8 after 3–4 days of incubation. Bourguignon et al. [25] described a CD44-dependent HYAL2 hyaluronidase activity curve optimal in the range of pH 4.5 to 5.5 with pH 6.8 being a less-optimal "shoulder" activity. Harada and Takahashi [28] described a CD44-dependent HYAL2 hyaluronidase activity curve optimal at pH 6.8 with no activity at pH 5.0. To determine whether hyaluronidase activity in chondrocytes represented shoulder or optimum activity, we measured CD44-dependent activity at pH 4.8. As shown in Fig. 6E, FITC-HA degradation at this lower pH could be observed starting at the earliest time point of 24 hours. Our works suggests that chondrocytes HYAL2 activity at pH 6.8 is more of a shoulder activity as observed in breast carcinoma cells [25]—a shoulder activity that is still active at pH 7.0. However, when FITC-HA was added directly to culture medium of C28/I2 cells (pH 7.4), no apparent degradation was observed after 4 days (Fig. 6C), unlike that observed by others in transfected HEK293 cells [28].

Cartilage extracellular matrix degradation is enhanced by cytokines such as IL-1β [30]. However, analysis of direct extracts of OA cartilage show the presence of CD44 fragmentation (Figure 1C), suggesting the activity of CD44 sheddases. We have previously shown increased CD44 shedding following treatment of chondrocytes with IL-1β [29] et al. 2010); herein we show the accumulation of HYAL2 in the media of chondrocytes treated with IL-1 β (Figures 1C, 2, 3A). We [31] and others [32] have shown, using human and bovine articular cartilage chondrocytes, that HYAL2 is the major hyaluronidase gene product expressed, but that there was no change in HYAL2 mRNA expression levels following IL-1β treatment. Thus, it is likely that IL-1 β induces the CD44 sheddases and release of HYAL2 from the cell surface.

HA turnover close to the chondrocyte cell surface is likely accomplished by CD44-mediated endocytosis of HA (and possibly HA and HYAL2) for delivery to lysosomes. The question arises as to how released or secreted HYAL2 could function farther removed from the chondrocytes such as within the interterritorial matrix wherein the pH may at times be low but not likely below 5.0. We hypothesize from the results of this study, that a fraction the HYAL2 present at the cell surface, while still complexed to CD44, is released when CD44 is enzymatically shed from chondrocytes. These shed HYAL2/CD44 complexes would then be able to migrate further into the extracellular matrix and more importantly, retain a limited but ultimately effective hyaluronidase activity within a near-neutral pH range. The regulation of these events would thus be dependent on the activity of cell surface sheddases such as MT1-MMP (MMP14), MT4-MMP (MMP17), ADAM10 or ADAM17 [33–36].

4. Experimental procedures

4.1. Cells and cell culture

Human articular chondrocytes were isolated from knee cartilage obtained following joint replacement surgery, within 24 hours after surgery and with institutional approval. These cartilage samples were from patients (50% female, 50% male) ranging in age from 56 to 74

years. Primary bovine articular chondrocytes were isolated from the articular cartilage of metacarpophalangeal joints of 18–24 month old adult bovine steers. Both human and bovine chondrocytes were liberated from full-thickness slices of articular cartilage by sequential 0.4% pronase and 0.025% collagenase P digestion (EMD Scientific). The chondrocytes were grown as high-density monolayers (2.0×10^6 cells/cm^2) in 1:1 Dulbecco's modified Eagle's medium (DMEM)/Ham's-F12 medium containing 10% fetal bovine serum (FBS; Hyclone) and 1% L-glutamine and penicillin-streptomycin. The immortalized human chondrocyte cell line, C28/I2 [37] (obtained from Dr. Mary Goldring, Hospital for Special Surgery, NY), was grown in DMEM containing 10% FBS. For immunostaining, C28/I2 cells were fixed with buffered 2% paraformaldehyde, permeabilized with 0.2% Triton-X-100, and incubated with the primary antibody; either CD44 cytotail [29] or the anti-human HYAL2 antibody (Sigma-Aldrich); detected with a FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) and mounted using Fluoro-Gel medium containing DAPI nuclear stain (Electron Microscopy Sciences).

4.2. Western blotting

Total protein was extracted using Cell-Lysis-Buffer (Cell Signaling) containing protease (ThermoScientific) and phosphatase (Sigma-Aldrich) inhibitor cocktails. Equivalent protein concentrations in the lysates (BCA; ThermoScientific) per lane were loaded into 4–12% NuPAGE® Novex® Tris-acetate gradient mini gels (Invitrogen); media samples were loaded by volume relative to the protein concentrations in the corresponding cell lysates. Some freshly obtained intact human OA cartilage was weighed, frozen and pulverized in liquid N_2 and then solubilized in RIPA buffer (Sigma-Aldrich) based on wet weight, for 30 min at 4 °C, prior to electrophoresis. Following electrophoresis, proteins within the acrylamide gel were transferred to a nitrocellulose membrane using a Criterion blotter apparatus (BioRad), and the nitrocellulose membrane was then blocked in 5% non-fat dry milk in Tris buffered saline containing 0.1% Tween 20 (TBS-T). Blots were probed with antibodies including: a peptide specific rabbit polyclonal antibodies that recognizes the C-terminal amino acids of the cytoplasmic tail of CD44 [cytotail antibody, prepared as shown previously [29]; an anti-bovine HYAL2 antibody (Santa Cruz) and; an anti-human HYAL2 antibody (Sigma-Aldrich). After incubation with corresponding secondary antibody, detection was performed using enhanced chemiluminescence reagents (Novex ECL; Invitrogen). X-ray films were then digitized using a Chemi-Doc Imager (Bio-Rad). Quantification of pixel intensity of bands was derived from 12-bit RAW images using Quantity One software (Bio-Rad) on a scale from 0–4096 pixels. Bar graphs were prepared to demonstrate mean fold change ± error (between replicate experiments) in pixel intensity relative to the pixel intensity of control bands (set = 1.0). In some cases, the actual digital scan of the gel lanes (pixel intensity versus R*f*) is shown for reference.

4.3. Co-Immunoprecipitation

C28/I2 cell monolayers were extracted using 10 mM Tris, pH 7.5 with 2 mM EDTA, 1% triton X-100 and protease and phosphatase inhibitors. Magnetic protein G Dynabeads® (Invitrogen) were used following the manufacturer's product protocol with minor modifications. Typically, between 500–1000 μg of protein lysate was incubated with antibody-Dynabead complex (10 μg of the anti-CD44 cytotail antibody [29, 38] as the

pulldown antibody diluted in 200 μl of PBS-Tween 20 suspension of magnetic beads) overnight at 4 °C [38]. The non-binding protein in the immunoprecipitate (termed the flow-through or “F” fraction) was collected for further analysis. The beads were washed three times, and the immunoprecipitated proteins were eluted from the beads-antibody-antigen complex using 20 μl of elution buffer (50 mM Glycine, pH 2.8). The eluted protein fraction is termed the bound or “B” fraction. The recovered protein fractions were mixed with 10 μl of NuPAGE® LDS sample buffer, 4 μl of reducing agent and 6 μl of deionized water, and incubated 10 minutes at 70°C and loaded into a 4–12% NuPAGE® Novex® Tris-acetate gradient mini gel and analyzed by western blot analysis. Several antibodies were conjugated with horse radish peroxidase (HRP) following the protocol for the Lightning-Link HRP conjugation kit (Innova Biosciences) to avoid cross-reactivity with immunoglobulins of other species in co-immunoprecipitation assays, by avoiding the use of secondary reagents. The HRP-conjugated antibodies included anti-CD44-cytotail-HRP and anti-HYAL2-HRP.

4.4. Hyaluronan zymography

Hyaluronidase activity present in cell lysates and concentrated conditioned media of chondrocytes cultures was assayed by HA-zymography as previously described [20]. The cell lysates were loaded by equal protein concentrations per lane whilst the media samples were loaded by volume relative to the protein concentrations in the corresponding cell lysates. Briefly, the samples were separated on a 8.5% SDS polyacrylamide gel containing 0.17 mg/ml HA (Genzyme) followed by a 2-hour incubation in 0.3% Triton X-100 at room temperature followed by an overnight incubation at 37°C in 0.1 M sodium formate pH 3.7 containing 0.15 M NaCl. Next, the gel was treated for 2 hours at 37°C with 1mg/ml pronase in 20 mM Tris HCl, pH 8.0 and then stained overnight with 0.015% Stains-All (Sigma-Aldrich) in a formamide buffer [39]. The gel was destained in distilled water until the background was sufficiently reduced to visualize the cleared, white bands. To test hyaluronidase activity at higher pH conditions, zymography was performed at pH 5.0, using bovine testicular hyaluronidase (Sigma-Aldrich) as a standard. The bands were directly imaged using a Chemi-Doc imaging system (Bio-Rad). Quantification of pixel intensity of bands was derived from 12-bit RAW images using Quantity One software (Bio-Rad) on a scale from 0–4096 pixels. Bar graphs were prepared to demonstrate mean fold change ± error (between replicate experiments) in pixel intensity relative to the pixel intensity of control bands (set = 1.0).

4.5. Detection of hyaluronan degradation by agarose gel electrophoresis

Hyaluronidase activity was also examined by visualizing the depolymerization of high molecular mass, fluorescein isothiocyanate-conjugated HA (FITC-HA) on 1% agarose electrophoresis gels. FITC-HA was prepared as described previously [40]. In the first approach, detection of hyaluronidase activity on a cell monolayer was studied. FITC-HA (200 μg/ml) was added to the media of 4 cultures of C28/I2 chondrocytes for 4 days and then visualized on a 1% agarose gel and detected under ultraviolet light. The samples were compared to a blank FITC-HA (Ctr) that was incubated for four days in the absence of a cell monolayer.

In a second approach, CD44-HYAL2 complexes were prepared from lysates of confluent cultures of C28/I2 cells and immunoprecipitated as described above using Magnetic Protein G Dynabeads®. Following overnight incubation at 4 °C the beads/complexes were collected, the supernatant discarded and beads washed twice in 100 μL of 200 μg/mL of FITC-HA in 50 mM NaCl, 100 mM phosphate buffer at pH 6.5 or pH 4.8. The beads were then resuspended in 100 μL of 200 μg/mL of FITC-HA at pH 6.8 or pH 4.8 and allowed to incubate in a 37°C with gentle rotational mixing. At 24 hour intervals, 15 μL aliquots were applied to a 1% agarose gel (IBI Scientific) in Tris-EDTA buffer (IBI Scientific) and electrophoresed for 30 minutes at 150 V. Fluorescent bands were visualized and imaged using transillumination of a fluorimaging system (Chemi-Doc). The gels were then fixed and stained with 0.015% Stains-All in formamide buffer, destained in water and re-imaged.

The extent of FITC-HA band migration visualized on agarose gels due to enzymatic cleavage was quantified using Quantity One densitometry software for n = 3 experiments at pH 6.8 and n = 2 experiments at pH 4.8. Pixel intensities of agarose gel lanes were derived from 12-bit RAW images on a scale from 0–4096. Side-by-side comparison digital scans of each lane of a particular agarose gel were shown as profiles depicting pixel intensities on the y-axis versus the R*f* on the x-axis. The mean fold change in R*f* value of a sample (taken at peak pixel intensity) as compared to the R*f* at peak pixel intensity of the control (set to 1.0 for each time point), was used to quantify the fold change in migration of the sample. An unpaired Student's *t*-test was used to evaluate the data and a p value of <0.05 was considered significantly different from control.

5. Conclusions

Experimental conditions that enhanced CD44 shedding from chondrocytes resulted in a proportional increase in HYAL2 released into the medium. By HA zymography, wherein enzymes are separated from all non-covalent partner proteins and co-factors, only activity at pH 3.7 was observed and this activity was reduced by pre-treatment of chondrocytes with trypsin. CD44 and HYAL2 were found to co-immunoprecipitate, and to co-localize intracellularly and at the plasma membrane. Weak hyaluronidase activity could be observed at pH 6.8 under assay conditions in which CD44 and HYAL2 binding remained intact. Additionally, hyaluronidase activity at pH 4.8 was clearly detected under these assay conditions which retained CD44-HYAL2 binding. This investigation suggests that CD44 and HYAL2 are bound at the surface of chondrocytes and this HYAL2 has the capacity for hyaluronidase activity. The release of HYAL2 when CD44 is shed could provide a mechanism for weak hyaluronidase activity to occur within the more distant extracellular matrix of cartilage.

Acknowledgements

The authors thank Dr. Larry J. Dobbs, Jr. of the Department of Pathology and Laboratory Medicine at East Carolina University for his assistance in the appropriation of human osteoarthritic tissue. The authors also thank Ms. Michelle Cobb and Ms. Kayla Britton for their technical assistance. A special thanks is given to Dr. Mary Goldring, Hospital for Special Surgery, NY for kindly providing the C28/I2 human chondrocytes. Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers R01-AR44384 (WK), R21 AR066581-01 (WK) and R01-

AR39507 (CBK). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Abbreviations

OA	osteoarthritis
MMP	matrix metalloproteinase
HA	hyaluronan
HYAL2	hyaluronidase 2
FITC	fluorescein isothiocyanate
GPI	glycosylphosphatidylinositol
WB	western blot
Tryp	trypsin-treatment
GM	GM6001

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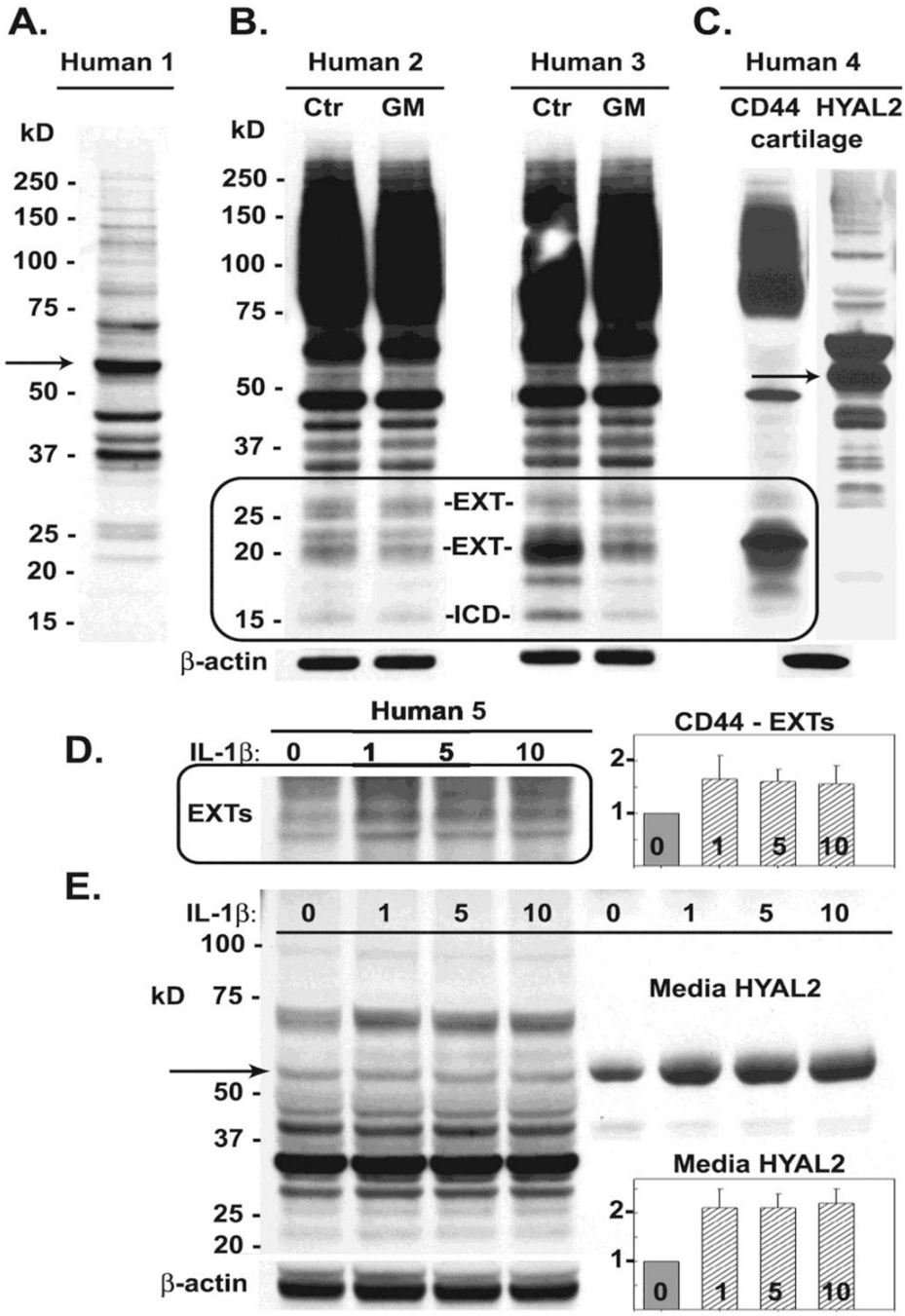

Figure 1. CD44 and HYAL2 protein expression in primary human chondrocytes

Panel A: HYAL2 protein was detected by western blotting in a human OA chondrocyte cell lysate; arrow depicts the principal 54 kD protein indicative of HYAL-2. Image shown is representative of lysates derived from n=6 patients. Panel B: Western blot profiles of CD44 protein in OA chondrocytes treated without (Ctr) or with GM6001 (GM); images shown are representative of lysates derived from n=4 patients. Panel C: CD44 (CD44 EXTs in boxed area) and HYAL2 protein (arrow) were detected by western blotting of direct OA cartilage extracts. Image shown is representative of direct cartilage extracts from n=6 patients. Panel

D: Low molecular mass CD44 fragments (EXTs in box) detected in cell lysates from an OA chondrocyte culture following treatment with 0 – 10 ng/ml IL-1β with relative quantification of these CD44-EXT bands from n=2 patients (bar graph; mean fold increase ± range, relative to control, no IL-1 β, set = 1.0). Panel E: HYAL2 protein in lysates from cell lysates from the same human OA chondrocyte culture as shown in panel D following treatment with 0 – 10 ng/ml IL-1β. HYAL-2 protein present in the medium fraction of these same cultures (Media) is shown at right as labeled. Arrow depicts the principal 54 kD protein indicative of HYAL-2. Relative quantification of these media HYAL2 bands from n=2 patients (bar graph; mean fold increase ± range, relative to control, no IL-1 β, set = 1.0). Panels show representative data from 5 different patients. Equivalency of lysate aliquots was verified by reprobing of the western blots for β-actin.

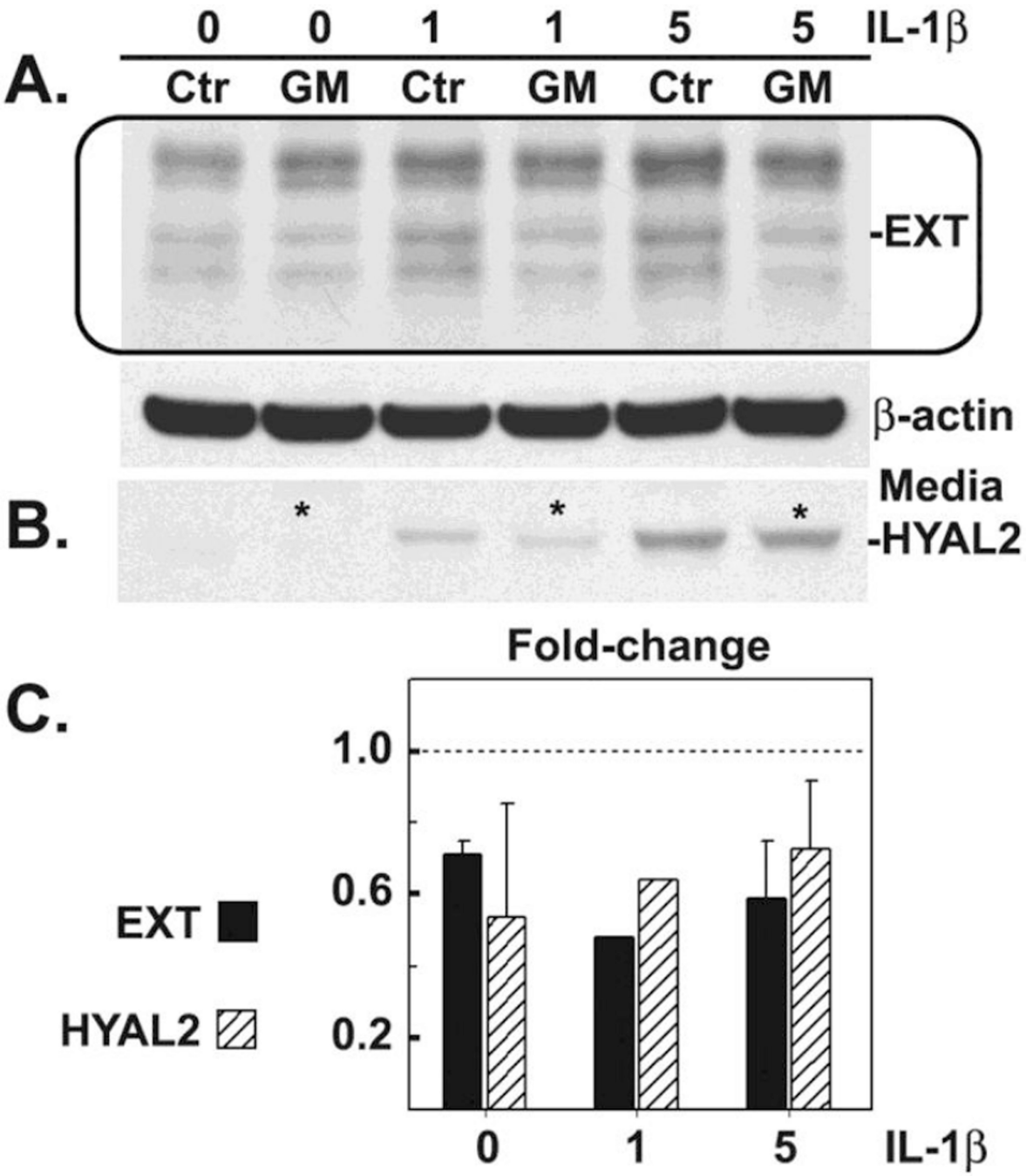

Figure 2. CD44 fragmentation and HYAL2 release in primary bovine articular chondrocytes

Panel A: CD44 EXTS (in boxed areas) were detected by western blotting of cell lysates of bovine chondrocytes treated with 0, 1 or 5 ng/ml IL-1β and, without (Ctr) or with co-treatment with GM6001 (GM). Equivalency of lysate aliquots was verified by reprobing of the western blots for β-actin. Panel B: HYAL2 protein was detected in the medium fraction (Media) of these same cultures, the principal 54 kD protein is indicative of HYAL-2. Panel C: Bar graph shows the fold-decrease in CD44 EXT (black bars) in the presence of GM as compared to Ctr (without GM; set = 1.0, dotted line) and the fold-decrease in HYAL2

(hatched bars) released into the media in the presence of GM as compared to Ctr (dotted line), with the co-treatment with 0, 1 or 5 ng/ml IL-1β. Bars show the mean (n = 2 experiments) ± range (error bars for 0 and 5 ng/ml conditions).

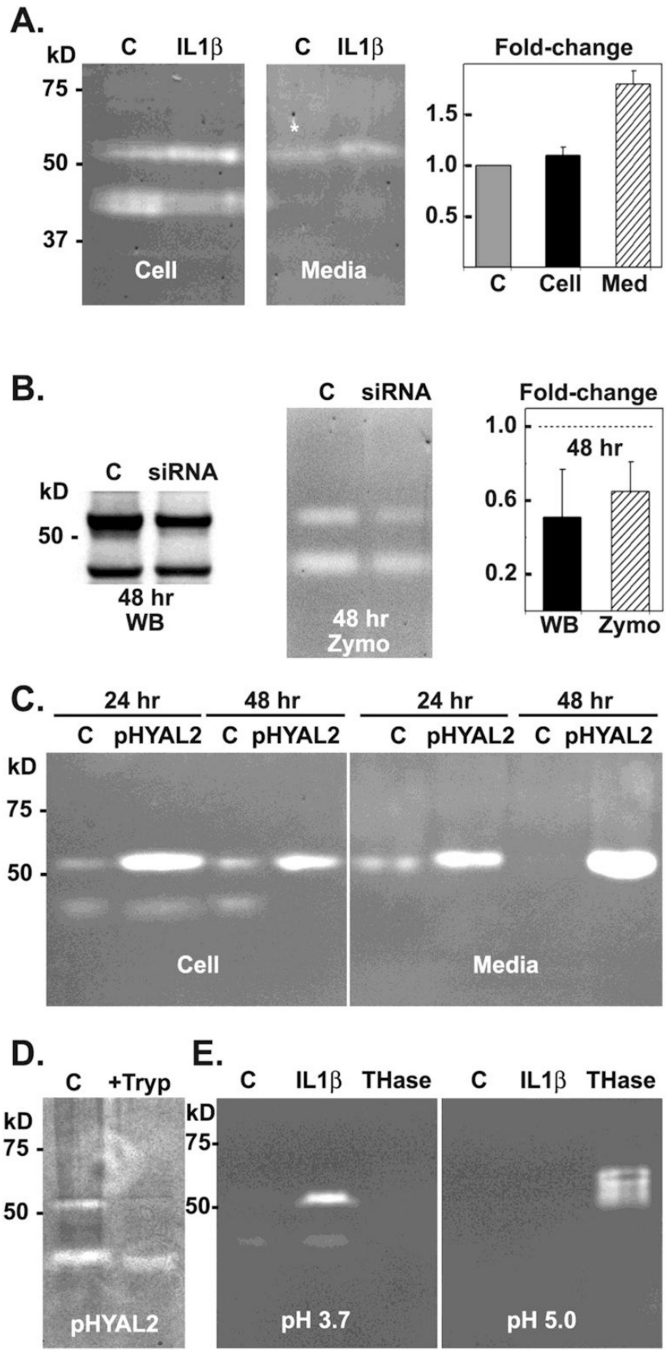

Figure 3. Detection of hyaluronidase activity using HA zymography

Panel A: Bovine articular chondrocytes in monolayer were cultured for 48 hrs without (C) or with 10 ng/ml IL-1β and aliquots of equivalent protein from cell lysates (cell) or equivalent volumes for media fractions were analyzed by HA zymography. Relative quantification of these hyaluronidase activity bands from n=2 experiments (bar graph) show mean fold increase ± range, relative to control, no IL-1 β, set = 1.0. Panel B: Cell lysates prepared from bovine chondrocytes transfected with control-siRNA (C) or HYAL2-specific siRNA (siRNA) and processed for western blotting (WB) to detect HYAL-2 protein; or HA

zymography (Zymo). Relative quantification of these hyaluronidase activity bands from n=2 experiments (bar graph) show mean fold increase ± range, relative to control (set = 1.0, dotted line), of HYAL2 (WB) or hyaluronidase activity. Panel C: Protein lysates and media were collected from bovine chondrocytes transfected with empty-vector (C) or pSPORT-HYAL2-vector (pHYAL2) 24 and 48 hrs after the transfection and equivalent protein aliquots analyzed by HA zymography. Panel D: Bovine chondrocytes transfected with pHYAL2 were treated without (C) or with 0.25% trypsin for 5 min (+Tryp) immediately prior to lysis and analysis by HA zymography. Panel E: Hyaluronidase activity in cell lysates from bovine chondrocytes stimulated without (C) or with IL-1β for 48 hrs, as well as 300 mU of bovine testicular hyaluronidase as a control (THase), were assayed by HA zymography under pH 3.7 and under pH 5.0 conditions. All zymograms depict cleared white bands on a stained HA substrate background.

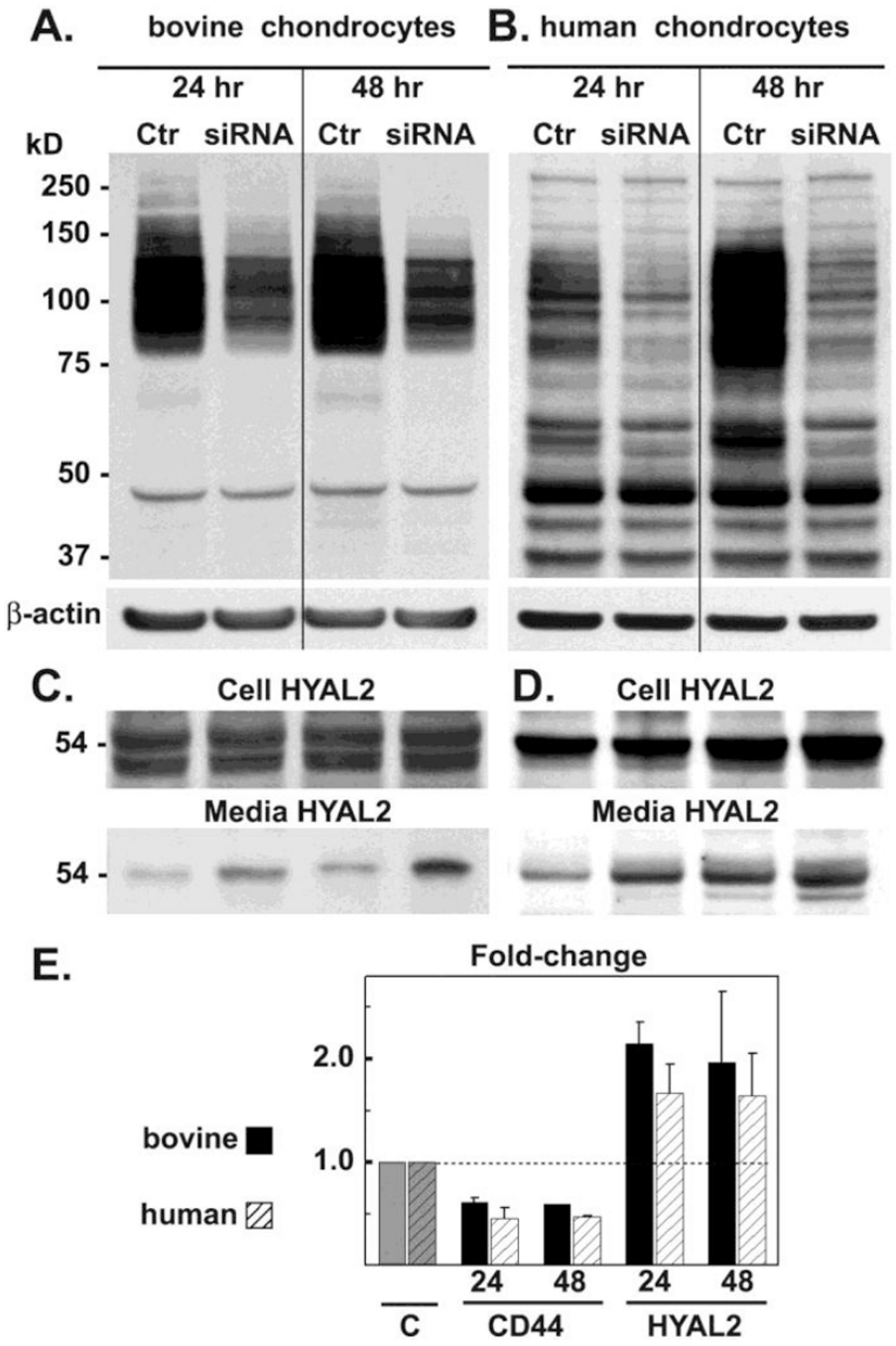

Figure 4. Effect of CD44 knockdown on HYAL2 release

To determine whether CD44 plays a role in HYAL2 retention, bovine chondrocytes (A,C) and human OA chondrocytes (B,D) were transfected with control siRNA (Ctr) or CD44-specific siRNA (siRNA) and then incubated in the presence of 1.0 ng/ml IL-1β for 24 and 48 hrs. At each time point, cell lysates were prepared and aliquots of equivalent protein processed by western blotting for the detection of CD44 (A,B). Equivalency of lysate aliquot loading was verified by reprobing of the western blots for β-actin. Cell lysates were also processed by western blotting to detect HYAL2 (C, D). Culture media samples were loaded

by volume relative to the protein concentrations in the corresponding cell lysates and processed by western blotting for the detection of HYAL-2 (C,D). Panel E: Relative quantification of these bands from n=2 experiments for bovine chondrocytes (black bars) and n=2 experiments for human chondrocytes (hatched bars) treated with CD44-specific siRNA show mean fold decrease ± range for CD44 and fold-increase ± range in media HYAL2 relative to control (C; set = 1.0).

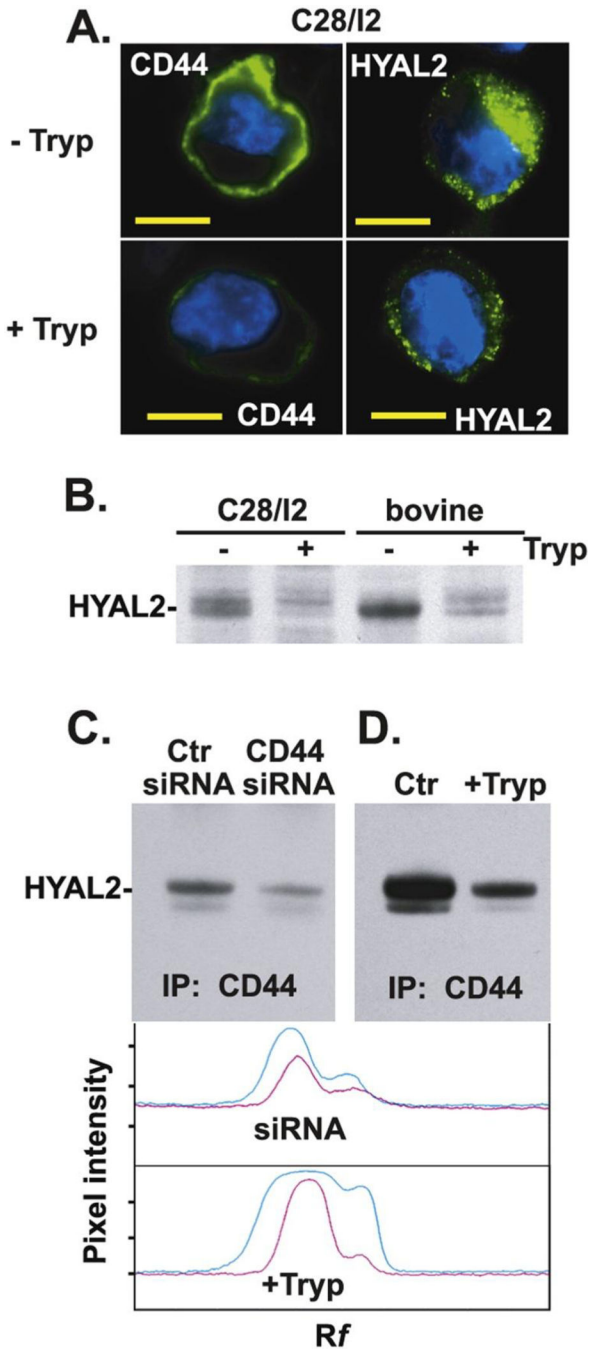

Figure 5. Localization and interaction of CD44 and HYAL2 in human C28/I2 chondrocytes

Panel A depicts the cellular localization of CD44 and HYAL2 via immunofluorescence of fixed and permeabilized C28/I2 cells. The C28/I2 cells were stained with or without pre-treatment with 0.25% trypsin (Tryp) for 5 min to delineate the extracellular localization of these two proteins—indicated by sensitivity to trypsin and reduction in staining. Images represent n=4 experiments. Panel B: C28/I2 cells and bovine chondrocytes also treated without (−) and with (+) trypsin, were analyzed by western blotting for the detection of HYAL2 protein. With trypsin pre-treatment a 65% decrease in HYAL2 was detected in

C28/I2 cells and a 63% decrease in HYAL2 was detected in bovine chondrocytes. To evaluate direct CD44 / HYAL-2 interactions, C28/I2 cells were transfected with control siRNA (Ctr siRNA) or CD44-specific siRNA (CD44 siRNA) and cell lysates immunoprecipitated using an anti-CD44 antibody. Panel C: Western blots of these cell lysates were probed for co-immunoprecipitating HYAL2 protein. Panel D: In other experiments, C28/I2 cells were treated without (Ctr) or with 0.25% trypsin (+Tryp) prior to the preparation of cell lysates, immunoprecipitation of CD44 and the probing of western blots for HYAL2 protein. Lower panels depict digital scans of pixel intensities of the entire length of the lanes (R*f*) in panel C and panel D using Quantity One software to quantify the HYAL2 in the immunoprecipitate in control (teal) or experimental (pink; siRNA or +Tryp) samples. The pixel intensity from 0 to 4096 is depicted on the y-axis (each tick is 1000 units) and the R*f* value on the x-axis.

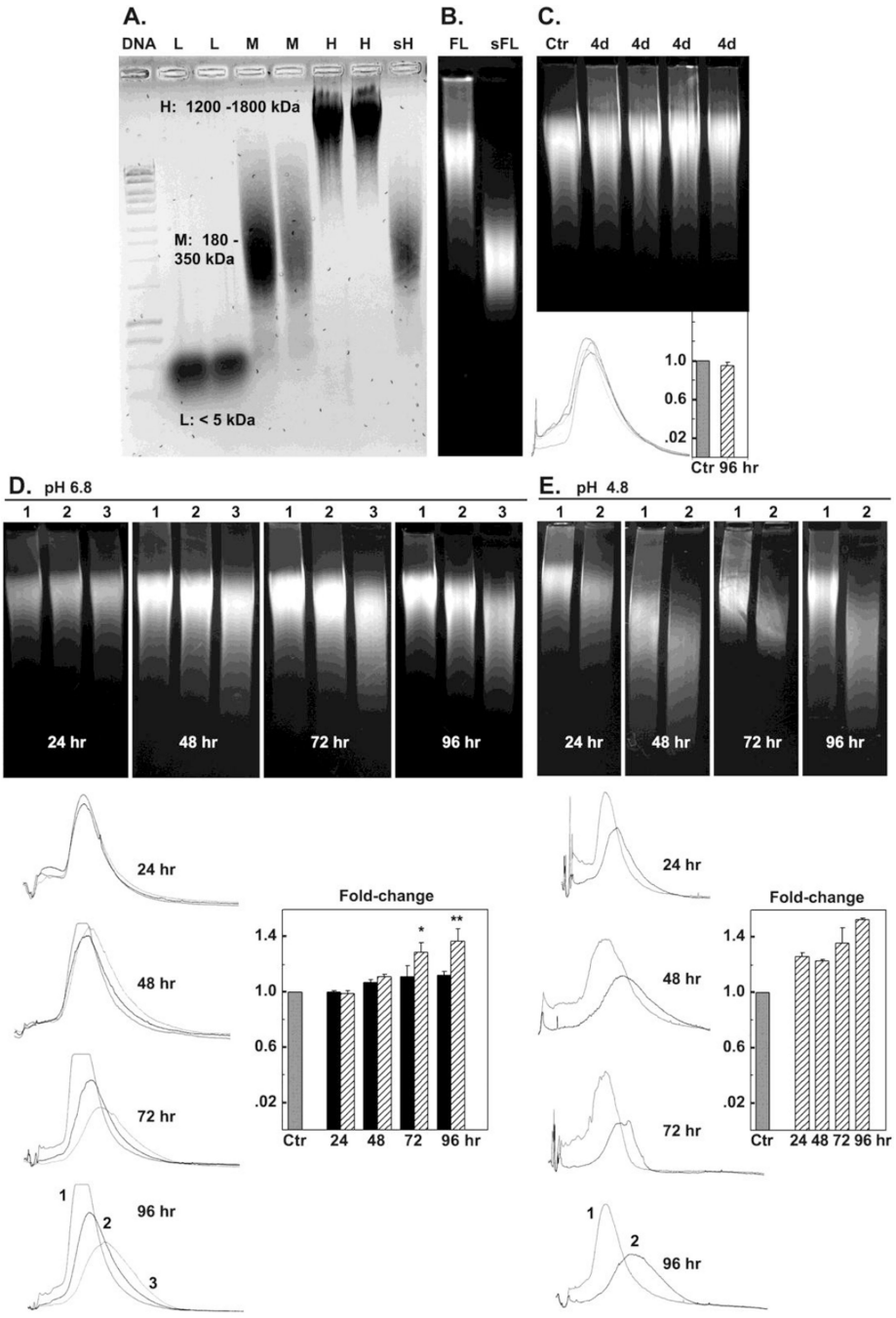

Figure 6. Detection of hyaluronidase activity using agarose gel electrophoresis

Panel A: Lanes depict low molecular mass HA standard of less than 5 kDa (L), middle molecular mass HA, 180–350 kDa (M), high molecular mass HA, 1200–1800 kDa (H) and high molecular mass HA treated by sonication (sH), and DNA ladder, all detected using Stains-All. Image shown is representative of 3 separate experiments. Panels B–E: gels visualized under ultraviolet light. Panel B: FITC-conjugation of high molecular mass HA, before (FL) and after sonication (sFL) were visualized. Image shown is representative of 3 separate experiments. Panel C: High molecular mass FITC-HA (200 μg/ml) was added to

the media of 4 cultures of C28/I2 cells for 4 days (4d) and then analyzed by agarose gel electrophoresis and compared to control FITC-HA (Ctr). Pixel intensities of the entire length of the lanes in the agarose gels were plotted on the y-axis and the Rf value on the x-axis. The bar graph depicts the mean fold change in peak pixel intensity after 96 hrs compared to control (set = 1.0). Panel D: 200 μg/ml aliquots of FITC-HA were incubated at 37 °C, pH 6.8, for indicated times; as FITC-HA only (lanes 1), with control Dynabeads (lanes 2) or with CD44/HYAL2 Dynabead complexes (lanes 3). Pixel intensities of the entire length of the lanes in the agarose gels were plotted on the y-axis and the Rf value on the x-axis. The bar graph depicts the mean fold change (for 3 separate experiments) in Rf value (taken at peak pixel intensity) of a samples as compared to the Rf at peak pixel intensity of the control (set = 1.0 for each time point, gray bar). Incubation of FITC-HA with control Dynabeads (black bars) or CD44/HYAL2 Dynabead complexes (hatched bars) are shown. * $p < 0.05$, ** $p < 0.01$ for comparison of CD44/HYAL2 Dynabead complexes to control Dynabead complexes. Panel E: 200 μg/ml FITC-HA was incubated at 37 °C, pH 4.8, for the indicated times; as FITC-HA only (lanes 1) or with CD44/HYAL-2 *Dynabead* complexes (lanes 3). Pixel intensities of the entire length of the lanes in the agarose gels were plotted on the y-axis and the Rf value on the x-axis. The bar graph depicts the mean fold change (for 2 separate experiments) in Rf value (taken at peak pixel intensity) of samples as compared to the Rf at peak pixel intensity of the control (set = 1.0 for each time point, gray bar). Incubation of FITC-HA with CD44/HYAL2 Dynabead complexes (hatched bars) are shown.