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High Resistance of the Mechanical Properties of the Chondrocyte Pericellular Matrix to Proteoglycan Digestion by Chondroitinase, Aggrecanase, or Hyaluronidase

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Abstract

In articular cartilage, the extracellular matrix (ECM) and chondrocyte-associated pericellular matrix (PCM) are characterized by a high concentration of proteoglycans (PGs) and their associated glycosaminoglycans (GAGs). These molecules serve important biochemical, structural, and biomechanical roles in the tissue and differences in their regional distributions suggest that different GAG/PG species contribute to the specific biomechanical properties of the ECM and PCM. The objective of this study was to investigate region-specific contributions of aggrecan, chondroitin and dermatan sulfate, and hyaluronan to the micromechanical properties of articular cartilage PCM and ECM *in situ*. Cryosections of porcine cartilage underwent digestion with ADAMTS-4, chondroitinase ABC, bacterial hyaluronidase or human leukocyte elastase. Guided by immunofluorescence for type VI collagen, AFM stiffness mapping was used to evaluate the elastic properties of matched PCM and ECM regions in paired control and digested cartilage sections. These methods were used to test the hypotheses that specific enzymatic digestion of GAGs or PGs would reduce both PCM and ECM elastic moduli. Elastase, which digests a number of PGs, some types of collagen, and non-collagenous proteins, was used as a positive control. ECM elastic moduli were significantly reduced by all enzyme treatments. However, PCM micromechanical properties were unaffected by enzymatic digestion of aggrecan, chondroitin/dermatan sulfate, and hyaluronan but were significantly reduced by 24% following elastase digestion. Our results provide new evidence for high resistance of PCM micromechanical properties to PG digestion and suggest a potential role for elastase in the degradation of the ECM and PCM.

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Keywords

Atomic force microscopy; type VI collagen; aggrecanase; chondroitin sulfate; chondron; perlecan; hyaluronic acid

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Introduction

Under normal conditions, the extracellular matrix (ECM) of articular cartilage is maintained in a slow state of turnover by a balance between the anabolic and catabolic activities of the chondrocytes (Mueller and Tuan, 2011). One of the defining characteristics of the cartilage ECM is its high concentration of proteoglycans (PGs) that comprise 4 – 8% of the tissue's wet weight (Hardingham and Fosang, 1992; Heinegard, 2009). PGs and their associated glycosaminoglycans (GAGs) interact with various collagens and other non-collagenous proteins and serve important biochemical and structural roles in the cartilage ECM (Roughley, 2006). The type and abundance of PGs in cartilage show significant spatial inhomogeneities in the tissue, with a higher concentration of these molecules found in the chondrocyte-associated pericellular matrix (PCM) than in the ECM (Poole et al., 1984).

In the ECM, the most prominent PG is aggrecan, which is found in large macromolecular aggregates with a hyaluronan (HA) backbone stabilized by link protein (Morgelin et al., 1988). Aggregates fill the spaces between type II collagen fibers and generate a fiber-reinforced composite through interactions with the collagen network (Mow et al., 1992). Due to its high abundance and fixed negative charge-associated with its chondroitin sulfate (CS) and keratan sulfate GAG chains, aggrecan is the main contributor to the macroscale osmotic swelling and low permeability that confer the tissue's bulk compressive properties (Eisenberg and Grodzinsky, 1985; Lai et al., 1991; Mow et al., 1998; Sun et al., 2004). Additional PGs in the ECM, including decorin, a dermatan sulfate (DS)/CS PG, and type IX collagen, a CS PG, interact with type II collagen fibers and are thought to organize the collagen network and regulate inter-fiber spacing and sliding (Hedbom and Heinegard, 1993; Scott and Stockwell, 2006; Wu et al., 1992). While the influence of these PGs and their associated GAGs on macroscale tensile, compressive, and shear properties of cartilage have been evaluated (Asanbaeva et al., 2008; Hall et al., 2009; Schmidt et al., 1990; Zhu et al., 1993), their influence on the microscale properties of the tissue has yet to be determined.

The PCM is a narrow tissue region that surrounds every cell in cartilage and is hypothesized to serve important roles as a biomechanical and biochemical transducer for chondrocytes (Guilak et al., 2006). While often defined by the exclusive presence of type VI collagen (Alexopoulos et al., 2009; Poole et al., 1988), the cartilage PCM is also characterized by a high concentration of PGs and GAGs relative to the ECM (Hunziker et al., 2002; Poole et al., 1984), including aggrecan monomers and small aggregates (Poole et al., 1982), HA (Knudson, 1993), type IX collagen (Hu et al., 2006; Poole et al., 1997), and the exclusive presence of other PGs, including biglycan (Kavanagh and Ashhurst, 1999) and perlecan (SundarRaj et al., 1995). Many of these PG components, most notably biglycan and HA, are hypothesized to play a role in PCM organization through their interactions with type VI collagen. Biglycan, a DS/CS PG, has been shown to strongly interact with and facilitate

network assembly of type VI collagen (Wiberg et al., 2001; Wiberg et al., 2002) and may contribute to PCM integration with the ECM by mediating interactions between type VI collagen and type II collagen and aggrecan (Wiberg et al., 2003). HA, in addition to serving as the backbone of newly assembled aggrecan aggregates, is found in the PCM as an independent GAG where it influences matrix organization through interactions with CD44 (reviewed in (Knudson, 2003)) and type VI collagen microfilaments (Kielty et al., 1992; McDevitt et al., 1991). In a recent study, we demonstrated a biomechanical role for the heparan sulfate chains of perlecan in the PCM and that selective enzymatic digestion of heparan sulfate alters PCM biomechanical properties *in situ* with minimal effect on the surrounding ECM (Wilusz et al., 2012). Furthermore, perlecan can serve as a pericellular depot for the heparin-binding growth factor fibroblast growth factor 2 (FGF-2), which can be released upon loading or injury to inhibit the activity of aggrecanases ADAMTS-4 and ADAMTS-5 (Vincent et al., 2007). These findings illustrate that GAGs and PGs are significant contributors to PCM microscale mechanical properties and suggest that other species may also play important biomechanical roles in this region. Importantly, all enzymes and ECM macromolecules secreted by the chondrocytes must pass through the PCM, where they may be retained or modified (Guilak et al., 2006; Melrose et al., 2008). Thus, an understanding of the influence of various PG-degrading enzymes on the mechanical properties of the PCM and ECM could provide important insights into the functional properties of these matrix regions under normal or pathologic conditions.

The objective of this study was to investigate region-specific contributions of aggrecan, CS/DS, and HA to the micromechanical properties of articular cartilage PCM and ECM. To this end, cryosections of porcine cartilage were subjected to specific enzymatic digestion with aggrecanase (ADAMTS-4), chondroitinase ABC (C-ABC), or bacterial hyaluronidase (Hyal). A newly developed method for immunofluorescence-guided atomic force microscopy (AFM) was used to quantify the elastic properties of matched PCM and ECM regions in paired control and digested cartilage sections based on the presence of type VI collagen (Wilusz et al., 2012). These methods were used to test the hypotheses that enzymatic digestion of PGs would reduce both PCM and ECM elastic moduli and to determine the specific contribute of CS, aggrecan, and HA to these properties. As a positive control, the effects of broad-spectrum enzymatic digestion with human leukocyte elastase were determined on both ECM and PCM mechanical properties.

Methods

Tissue Sample Preparation

Full thickness articular cartilage samples were harvested from the medial condyles of 2 – 3 year old, skeletally mature, porcine knee joints exhibiting no signs of macroscopic degeneration. Cartilage samples were wrapped in phosphate-buffered saline (PBS)-soaked gauze and frozen at -20°C for intermediate storage. Samples were embedded in water-soluble embedding medium (Tissue-Tek O.C.T. Compound, Sakura Finetek USA, Inc., Torrance, CA) and sectioned perpendicular to the articular surface in 5 µm-thick sections using a cryostat microtome (Leica CM1830; Leica Microsystems, Inc., Buffalo Grove, IL).

Cartilage sections were collected on glass slides and washed thoroughly with PBS to remove the embedding medium prior to further treatment.

Specific Enzymatic Digestions with ADAMTS-4, Chondroitinase ABC, and Bacterial Hyaluronidase

Cartilage sections were incubated in 50 μ L of 0.04 mU/mL recombinant human ADAMTS-4 (aggrecanase-1; EC 3.4.24.82; Anaspec, Inc., San Jose, CA) in 50 mM Tris-HCl (Sigma-Aldrich, St. Louis, MO) containing 150 mM sodium chloride (EM Science, Gibbstown, NJ), 5 mM calcium chloride (EM Science), pH 7.5 at 37°C for 60 minutes. Undigested control sections from the same cartilage specimens were incubated at 37°C for 60 minutes in ADAMTS-4 enzyme buffer (0 mU/mL). As ADAMTS-4 and ADAMTS-5 primarily cleave aggrecan at the same site (Glu³⁷³-Ala³⁷⁴) (Westling et al., 2002), only ADAMTS-4 was examined in the present study.

Chondroitinase ABC (C-ABC) (from *Proteus vulgaris*, EC 4.2.2.4; Sigma-Aldrich) has demonstrated activity toward chondroitin-4-sulfate (C-4-S, chondroitin A), DS (chondroitin B), and chondroitin-6-sulfate (C-6-S, chondroitin C) (Yamagata et al., 1968). Cartilage sections were incubated in 50 μ L of 0.25 U/mL C-ABC solution in 50 mM Tris (Sigma-Aldrich) containing 60 mM sodium acetate (Sigma-Aldrich) and 0.02% bovine serum albumin (BSA; Invitrogen, Life Technologies, Grand Island, NY), pH 8.0 at 37°C for 30 minutes. At this higher pH, C-ABC demonstrates little activity toward HA (optimal pH 6.8) (Yamagata et al., 1968). Undigested control sections from the same cartilage specimens were incubated at 37°C for 30 minutes in C-ABC enzyme buffer (0 U/mL).

Hyaluronidase (Hyal) (from *Streptomyces hyalurolyticus*; EC 4.2.2.1; Sigma-Aldrich) is specific for HA and is distinct from other hyaluronidases in that it is inactive toward chondroitin derivatives (Ohya and Kaneko, 1970). Cartilage sections were incubated in 50 μ L of 60 U/mL Hyal solution in 20 mM phosphate buffer containing 77 mM sodium chloride (EM Science) and 0.01% BSA (Invitrogen), pH 6.0 at 37°C for 30 minutes. Undigested control sections from the same cartilage specimens were incubated at 37°C for 30 minutes in Hyal enzyme buffer (0 U/mL).

Broad Spectrum Digestion with Human Leukocyte Elastase

Cartilage sections were incubated in 50 μ L of 1 U/mL human leukocyte elastase (EC 3.4.21.37; Sigma-Aldrich) solution in 20 mM Tris (Sigma-Aldrich) containing 10 mM calcium chloride (EM Science), pH 8.0 at 37°C for 30 minutes. Undigested control sections from the same cartilage specimens were incubated at 37°C for 30 minutes in elastase enzyme buffer (0 U/mL).

Immunofluorescence for Type VI Collagen

Following digestion, cartilage sections were labeled for type VI collagen using a modified immunofluorescence protocol (Wilusz et al., 2012; Youn et al., 2006). Sections were blocked in 10% normal donkey serum (Lot #: S10011325; Fitzgerald Industries International, Acton, MA) diluted in assay buffer (0.1% BSA (Invitrogen) in 0.1 M TBS, pH 7.3) for 20 minutes at room temperature. Samples were incubated with primary antibody for

type VI collagen (anti-collagen type VI raised in rabbit, 70R-CR009X; Fitzgerald) at a 1:300 dilution in 10% donkey serum for 20 minutes at room temperature. After two washes of 5 minutes each, samples were incubated with secondary antibody (FITC-conjugated donkey anti-rabbit IgG, 43R-ID0671FT; Fitzgerald) at a 1:200 dilution in 10% donkey serum for 20 minutes in the dark at room temperature. Sections were rinsed twice in TBS for 5 minutes each and remained in TBS at room temperature during AFM testing. No significant alteration in type VI collagen labeling was observed with any enzyme treatment.

Mechanical Characterization via AFM Stiffness Mapping

AFM stiffness mapping (Darling et al., 2010) was performed using simultaneous force measurements and fluorescence imaging with an AFM system (MFP-3DBio; Asylum Research, Santa Barbara, CA) integrated with an inverted fluorescence microscope (AxioObserver A1; Carl Zeiss, Inc., Thornwood, NY) as described previously (Wilusz et al., 2012) (Figure 1). For microscale indentation, borosilicate glass spheres (5 μm diameter) were attached to tip-less AFM cantilevers ($k = 4.5 \text{ N/m}$; Novascan Technologies, Ames, IA). Indentations were applied with a force trigger of 300 nN and curves were sampled at 7.5 kHz.

For evaluation of PCM elastic properties, 1600 indentations (15 $\mu\text{m/s}$ indentation velocity) were sequentially applied over each $20 \mu\text{m} \times 20 \mu\text{m}$ region of interest defined by microscopic examination with phase contrast imaging and positive immunofluorescence labeling for type VI collagen around cell-sized voids in the tissue section. Elastic properties of the adjacent ECM were evaluated using a similar approach applying 16 indentations over $20 \mu\text{m} \times 20 \mu\text{m}$ scan regions visually devoid of PCM and type VI collagen labeling (15 $\mu\text{m/s}$ indentation velocity). For all samples, AFM testing was completed within 4 hours of initial sectioning.

PCM and ECM microscale mechanical properties were evaluated in paired PCM/ECM scan regions in the middle/deep zone (200 – 400 μm from the articular surface) of cartilage sections digested with ADAMTS-4 ($N = 3$ joints, $n = 9$ total regions per treatment), C-ABC ($N = 6$ joints, $n = 24$ total regions per treatment), Hyal ($N = 6$ joints, $n = 24$ total regions per treatment), or elastase ($N = 3$ joints, $n = 12$ total regions per treatment).

Data Analysis

Raw data for z-piezo movement and cantilever deflection were collected and analyzed using a custom Matlab script (The MathWorks, Natick, MA). Elastic moduli were determined by fitting a modified Hertz model to collected force-indentation curves as described previously (Darling et al., 2010; Guo and Akhremitchev, 2006) (Figure 1D, E). The local Poisson's ratio was assumed to be 0.04 for both the ECM (Athanasίου et al., 1995) and PCM (Alexopoulos et al., 2005) in both control and digested samples. Hertzian contact mechanics provided excellent fits to the experimental data for all force-indentation curves ($R^2 > 0.90$). Two-dimensional contour maps were generated of the spatial distribution of calculated elastic moduli in each region. For clarity of comparisons, contour maps presented for each enzyme treatment are plotted on the same graded color scale.

The cartilage PCM was defined based on positive immunofluorescence labeling for type VI collagen around cell-sized voids and data were included for all indentations that fell within labeled regions as described previously (Wilusz et al., 2012; Wilusz et al., 2012). To quantitatively evaluate the spatial distribution of moduli in the chondrocyte microenvironment, the progression of elastic moduli from the PCM outer edge to the ECM was averaged over each radial increment of 0.5 μm .

Histological Staining

To visualize global loss of GAGs/PGs with each enzymatic digestion, histological staining was performed using Accustain Safranin-O solution (Sigma-Aldrich) and 0.02% aqueous fast green (Sigma-Aldrich) (Figure 2). Control experiments confirmed that no discernible loss of staining was observed between cryosections that underwent minimal washing following collection and undigested control sections incubated as described in each enzyme buffer (data not shown).

Statistical Analyses

For each enzyme treatment, the effect of digestion on ECM and PCM elastic moduli was evaluated using a two-way ANOVA (region, digestion; $\alpha = 0.05$) and Fisher's least significant difference (LSD) *post-hoc* test. When required, data were log-transformed for normality. All data presented as mean $\pm$ standard error.

Results

Histological staining confirmed a loss of Safranin-O staining with all enzyme treatments (Figure 2). In the ECM, ADAMTS-4 digestion resulted in a more moderate loss of staining as compared to the complete loss observed following digestion with C-ABC, Hyal, and elastase. In the PCM, GAG/PG staining remained in the chondrocyte microenvironment throughout the tissue depth following ADAMTS-4 and Hyal digestion. In contrast, C-ABC and elastase digestion resulted in a complete loss of PCM GAG/PG staining.

Specific enzymatic digestion with ADAMTS-4, C-ABC, or Hyal resulted in significant alterations in ECM, but not PCM, elastic moduli (Figure 3A, B, C; Figures 4-6). ECM elastic moduli were reduced by 30% following ADAMTS-4 digestion ($p < 0.01$), by 37% following C-ABC digestion ($p < 0.0005$), and by 32% following Hyal digestion ($p < 0.00005$) as compared to undigested controls. On the other hand, PCM moduli were unaffected by these enzyme treatments ($p > 0.30$). ECM moduli were significantly greater than PCM moduli in all undigested controls ($p < 0.05$) and following ADAMTS-4 and Hyal digestion ($p < 0.05$; Figure 3A, B; Figure 5A, B). In contrast, the mechanical distinction between the ECM and PCM was lost following C-ABC digestion ($p = 0.48$). Spatial mapping of elastic moduli revealed distinct differences in the chondrocyte microenvironment following digestion. In undigested control sections, elastic moduli exhibited a trend toward a significant effect of distance from the PCM outer edge in the ADAMTS-4 experiment group (Figure 4E; $p = 0.07$) and a significant effect in the C-ABC (Figure 5E), and Hyal (Figure 6E) experimental groups ($p < 0.05$). In contrast, following digestion a significant effect of distance was observed only in elastic moduli in Hyal

digested sections ($p < 0.05$). In all groups, control moduli were significantly greater than digested moduli at all distances outside the PCM ($p < 0.05$).

Broad spectrum enzymatic digestion with elastase significantly reduced both ECM and PCM biomechanical properties (Figure 3D, Figure 7). PCM elastic moduli were reduced by 24% following digestion as compared to undigested controls ($p < 0.05$; Figure 7E). ECM elastic moduli were reduced by 57% with elastase digestion as compared to controls ($p < 0.000001$). While ECM elastic moduli were significantly greater than PCM moduli in undigested controls ($p < 0.00005$; Figure 7A), mechanical distinction between the ECM and PCM was lost following elastase digestion ($p = 0.43$; Figure 7B). This result was also reflected in the modulus progression outward from the PCM outer edge (Figure 7E). In contrast to undigested control sections, elastase digested cartilage demonstrated no variation in elastic moduli with distance from the PCM ($p = 0.32$) and exhibited significantly reduced elastic moduli at all distances outside the PCM ($p < 0.05$).

Discussion

The micromechanical properties of the PCM exhibited high resistance to enzymatic disruption of aggrecan, CS/DS, and HA. Guided by immunofluorescence labeling for type VI collagen to mark the boundaries of the PCM, AFM stiffness mapping revealed no change in PCM elastic moduli following digestion with ADAMTS-4, C-ABC, or Hyal despite significant reductions in ECM moduli. On the other hand, significant reductions in both PCM and ECM moduli were observed following leukocyte elastase. These findings are in contrast to our original hypothesis that specific digestion of PGs or GAGs would significantly alter the mechanical properties of the PCM.

Our results suggest that the micromechanical properties of the PCM exhibit high resistance to enzymatic degradation of aggrecan, CS/DS, and HA. The lack of alteration in PCM properties was not due to a lack of activity of the enzyme preparations, as demonstrated by the concurrent loss of Safranin-O staining and significant decrease in ECM moduli in digested cartilage sections. In general agreement with our findings, previous studies have shown an absence of aggrecanase-generated aggrecan G1 fragments in the PCM and territorial matrix regions in normal articular cartilage, despite abundant epitope staining in the ECM (Lark et al., 1997) and constitutive production and activity of aggrecanases in the tissue (Tortorella et al., 2001). *In vitro*, C-ABC digestion failed to disrupt type VI collagen hexagonal networks pre-formed in the presence of intact DS/CS-substituted forms of biglycan (Wiberg et al., 2002). If a similar mechanism governs PCM assembly *in situ*, removal of DS chains from biglycan would have minimal effects on the structural and mechanical integrity of the PCM type VI collagen network. With regard to HA, no discernible change in PCM morphology or PG content was observed following up to 10 hours of digestion with ovine testicular hyaluronidase (Poole et al., 1985), which has demonstrated activity toward both HA and chondroitin derivatives (Menzel and Farr, 1998). Similarly, other studies have shown little effect of Hyal or interleukin 1 α treatment on collagen VI distribution of PCM architecture in articular cartilage (Jansen et al., 2010). However, collagenase digestion of articular cartilage completely degrades the ECM but leaves the chondron (chondrocyte and its PCM) intact, although the mechanical properties of

the PCM are greatly reduced in this case (Guilak et al., 1999) as compared to the *in situ* properties (Darling et al., 2010) and those of mechanically isolated chondrons (Guilak et al., 2005).

The mechanism behind this observed PCM-specific resistance has yet to be elucidated. One possible candidate is heparan sulfate, which along with heparin, has demonstrated an ability to inhibit the activity of many enzymes *in vitro*, including aggrecanases (Munteanu et al., 2002), C-ABC (Nakada and Wolfe, 1961), and hyaluronidases (Mio and Stern, 2002). In articular cartilage, heparan sulfate is unique to and highly concentrated in the chondrocyte microenvironment, where it is associated with syndecans on the cell surface (Pap and Bertrand, 2013) and perlecan in the PCM (Melrose et al., 2006; Wilusz et al., 2012). This localization provides a mechanism for direct interaction (Munteanu et al., 2002) and charge associated effects (Mio and Stern, 2002; Nakada and Wolfe, 1961) that inhibit enzyme activity specifically within the PCM. *In vivo*, the pericellular perlecan can bind and release FGF-2, which can in turn activate the production of enzyme inhibitors by chondrocytes (Chia et al., 2009; Vincent et al., 2007).

In agreement with previous studies that demonstrate a loss of cartilage macroscale properties following GAG depletion with C-ABC (Korhonen et al., 2003; Laasanen et al., 2003; Lyyra et al., 1999; Zhu et al., 1993) and various hyaluronidases (Zhu et al., 1993), porcine cartilage in the current study exhibited a 30% – 37% reduction in ECM microscale elastic moduli following digestion with ADAMTS-4, C-ABC, and Hyal. The observed effects of C-ABC digestion are likely dominated by the loss of CS chains from aggrecan rather than DS chains from decorin as a recent study demonstrated no effect of specific digestion of DS with chondroitinase B on cartilage GAG content or macroscale indentation properties (Hall et al., 2009). Interestingly, all three enzymes exhibited similar effects on ECM mechanical properties despite their different mechanisms of aggrecan disruption. ADAMTS-4 cleavage of the aggrecan core protein has been mapped to five sites (Sandy et al., 1991; Tortorella et al., 2000). C-ABC acts directly on the CS GAG chains, reducing their length through cleavage of the bonds between adjacent disaccharide units (Ernst et al., 1995), leaving the PG core protein intact. Hyal digestion disrupts aggrecan macromolecular aggregates via cleavage of the HA backbone releasing aggrecan monomers, link protein, and HA fragments from the tissue in the absence of aggrecan proteolysis (Durigova et al., 2011). Since interstitial fluid load support is minimal during AFM microindentation (Bonnevie et al., 2012; Park et al., 2009), our results suggest that disruption of aggrecan, regardless of the mechanism, significantly reduces the intrinsic properties of the solid matrix in proportion to its relative abundance [30 – 40% dry weight (Mow et al., 1992)].

The observed reduction in ECM mechanical properties is likely also related to the triphasic effects associated with the loss of GAG-associated fixed charge density. The fixed charge density is the primary contributor to the Donnan osmotic pressure, the main force behind cartilage swelling behavior. The Donnan osmotic pressure contributes 30 – 50% of the compressive aggregate modulus of articular cartilage (Eisenberg and Grodzinsky, 1985; Lai et al., 1991; Mow et al., 1998; Sun et al., 2004) and approximately 20% of the equilibrium Young's modulus at physiological ionic strength (Eisenberg and Grodzinsky, 1985; Lai et al., 1991; Mow et al., 1998; Sun et al., 2004). The observed 30 – 37% reduction in

microscale ECM moduli with digestion, coupled with the loss of ECM Safranin-O staining, is consistent with a significant loss of fixed charge density and Donnan osmotic pressure.

As shown by a number of theoretical and experimental studies, cartilage mechanical behavior is defined by complex interactions among the solid, fluid, and ionic phases of the tissue (Akizuki et al., 1986; Lai et al., 1991; Nagel and Kelly, 2010). In this respect, the mechanical properties of the tissue would be expected to be highly sensitive to alterations in the GAG content and GAG-collagen interactions caused by enzymatic digestion of the charged components, particular in regions with highly aligned collagen fibers (Nagel and Kelly, 2010). Thus the fact that enzymatic digestion of the GAGs resulted in significant alterations in ECM but not PCM properties provides further support for the finding that the PCM properties are highly resistant to these enzymes. Further studies examining these properties under different osmotic conditions could provide further insight into the relative contributions of the different charged and uncharged components to the mechanical properties of the PCM.

Elastase was the only enzyme treatment in the current study to significantly reduce both PCM and ECM micromechanical properties. The observed 57% reduction in ECM microscale moduli in the current work is comparable to previous studies at the macroscale (Bader and Kempson, 1994; Menninger et al., 1981) and microscale (Stolz et al., 2004). The larger reduction in ECM moduli following elastase digestion as compared to specific aggrecan-targeted digestions is likely related to the wide range of substrates cleaved by elastase, including aggrecan (Mok et al., 1992), small PG core proteins (Owen and Campbell, 1999), link protein (Mok et al., 1992), type VI collagen (Kielty et al., 1993), type IX and XI collagen (Gadher et al., 1988), and type II collagen cross-links but not triple-helical type II collagen (Gadher et al., 1988; Starkey et al., 1977). The reduction in PCM properties was likely driven by disruption of the type VI collagen network through direct cleavage of type VI collagen (Kielty et al., 1993) or disruption of biglycan-mediated type VI collagen interactions (Wiberg et al., 2001; Wiberg et al., 2002; Wiberg et al., 2003). Type VI collagen is a defining factor in both the biochemical and biomechanical definitions of the PCM (Alexopoulos et al., 2009; Poole et al., 1988). A previous study reported the mechanical properties of chondrons isolated from Col6a1 knockout mice that completely lack extracellular type VI collagen (Alexopoulos et al., 2009; Poole et al., 1988). PCM Young's moduli were significantly reduced in Col6a1^{+/-} and Col6a1^{-/-} mice, highlighting the important role of type VI collagen in PCM mechanical properties.

Our findings provide additional support for leukocyte elastase as a potential contributor to cartilage degeneration. Elevated levels of elastase are present in the synovial fluid of patients with rheumatoid arthritis (Elsaid et al., 2003; Ishiguro et al., 2001; Momohara et al., 1997), osteoarthritis (Elsaid et al., 2003; Momohara et al., 1997), and following acute joint injury (Elsaid et al., 2008; Elsaid et al., 2003). In addition to binding to the articular surface (Janoff et al., 1976; Kawabata et al., 1996), elastase is capable of diffusing through the cartilage matrix (Janoff et al., 1976) and binding to chondrocytes in a receptor-mediated manner (Bartholomew and Lowther, 1987; Menninger et al., 1981). These properties provide a means and potential mechanism for localization of elastase to PCM regions.

Digestion protocols employed in this study were optimized on cryosections of porcine cartilage to achieve measureable changes in ECM biomechanical properties and a loss of Safranin-O staining, which has been shown previously to exhibit a linear relationship with GAG content as measured by dimethylmethylene blue (DMMB) for articular cartilage (LeRoux et al., 2000; Martin et al., 1999). Incubation for 30 – 60 minutes was sufficient to achieve these aims for all enzyme treatments. The loss of GAG/PG staining was more moderate in ADAMTS-4 digested samples as compared to the other digestions. ADAMTS-4 cleaves faster within the CS attachment domains than within the interglobular domain, with detectable CS domain epitopes present after as little as 5 minutes of digestion as compared to interglobular domain epitopes which are detectable after 30 – 60 minutes (Tortorella et al., 2000). These site-to-site differences in efficiency likely contributed to the moderate loss of staining observed in the current work. Aggrecan monomers may have lost only a fraction of their CS chains as opposed to their entire GAG attachment domains, which could account for the similar mechanical effects observed among ADAMTS-4, C-ABC, and Hyal digested samples.

A number of proteolytic enzymes have been implicated in cartilage turnover and degeneration. In the present study, we focused on ADAMTS-4, C-ABC, *Streptomyces* Hyal, and leukocyte elastase because of their prevalence in previously published work characterizing the macroscale mechanical properties of PG/GAG depleted cartilage and the known roles of ADAMTS-4 and elastase in joint disease. ADAMTS-5 (aggrecanase-2) is another aggrecanase that has been implicated in cartilage degeneration (Song et al., 2007), has aggrecanase activity 1000-fold greater than ADAMTS-4 (Gendron et al., 2007), and has been shown to have a dominant role in aggrecan degradation in mice (Glasson et al., 2005). ADAMTS-5 is constitutively expressed by chondrocytes (Bau et al., 2002) and co-localizes with HA in the PCM in normal and OA cartilage (Plaas et al., 2007). Matrix metalloproteinases have a well-characterized role in cartilage degeneration and are known to cleave aggrecan (Flannery et al., 1992; Little et al., 2002) and small PGs (Monfort et al., 2006; Zhen et al., 2008). Other proteases associated with inflammation, including high temperature requirement A1 (HtrA1) and cathepsin B and L, are elevated in synovial fluid of patients with rheumatoid arthritis, osteoarthritis, or following joint injury (Lang et al., 2000; Solau-Gervais et al., 2007). These enzymes provide interesting targets for future studies to ascertain which, if any, alter the mechanical integrity of the PCM.

This study provides new evidence for high resistance of PCM micromechanical properties to aggrecan-targeted digestion but vulnerability to leukocyte elastase digestion. This resistance may be an important property of the chondrocyte micromechanical environment, which is highly dependent on the relative mechanical properties of the cell, the PCM, and the ECM (Choi et al., 2007; Julkunen et al., 2009; Kim et al., 2008; Mow et al., 1994). As alterations in PCM properties may have a significant influence on the biological response of the chondrocytes to loading, resistance of the PCM to enzymatic digestion may provide a mechanism to allow for enzyme transport from the chondrocyte to the ECM during normal matrix turnover without mechanical disruption of the PCM. However, our results suggest that the PCM is susceptible to proteolytic degradation by inflammatory cell-derived enzymes, like elastase, that have wide substrate specificity and provide new insight into the

relative roles of chondrocyte-mediated and inflammatory cell-mediated degradation in joint disease. Taken together with the observed PCM resistance to ADAMTS-4 digestion, these inflammatory cell enzymes may play an important role in the initiation and progression of degeneration of the chondrocyte micromechanical environment.

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Figure 1.

Immunofluorescence-guided atomic force microscopy (AFM) was used to map the elastic properties of matched PCM and ECM in cryosections of articular cartilage in paired control and digested samples. (A) Schematic of the AFM-fluorescence testing configuration. A borosilicate glass sphere (5 μm diameter) was attached to the AFM cantilever for testing. For evaluation of PCM elastic properties, 1600 indentations were sequentially applied over each $20\ \mu\text{m} \times 20\ \mu\text{m}$ region of interest defined by microscopic examination with (B) phase contrast imaging and (C) positive immunofluorescence labeling for type VI collagen around cell-sized voids in the tissue section. (D) Raw data for cantilever deflection and z-piezo position as recorded during indentation. Contact with the substrate is marked by a sharp increase in cantilever deflection in the approach curve (red). (E) Force-indentation data from the post-contact portion of the approach curve (red) is curve-fit with a modified Hertz model (black) to calculate the elastic modulus.

Figure 2.

Safranin-O (red, GAGs/PGs) and fast green (blue, collagens) of cartilage sections from paired undigested control (A, E, I, M) and digested (B, F, K, N) sections for ADAMTS-4, C-ABC, Hyal, and elastase digestion. Loss of ECM GAG/PG staining was observed with all digestions, with more moderate loss observed following ADAMTS-4 digestion. PCM GAG/PG staining remained following ADAMTS-4 (C, D) and Hyal (K, L) digestion and completely lost with C-ABC (G, H) and elastase (O, P) digestions. Representative images are shown for each enzyme treatment and PCM regions were imaged in the middle/deep zone (200 – 400 μm from the articular surface). ECM scale bar = 250 μm . PCM scale bar = 10 μm .

Figure 3.

Elastic moduli of ECM and PCM regions in undigested control (black) and digested (white) cartilage sections treated with (A) ADAMTS-4, (B) C-ABC, (C) Hyal, or (D) elastase. Moduli presented as mean + standard error. ADAMTS-4 – a: $p < 0.01$ for control ECM moduli as compared to ADAMTS-4 digested ECM moduli. b: $p < 0.05$ for ECM moduli as compared to respective PCM moduli. PCM moduli were unaffected by ADAMTS-4 digestion ($p = 0.30$) (N = 3 pigs, n = 9 regions per treatment). C-ABC – c: $p < 0.0005$ for control ECM moduli as compared to C-ABC digested ECM moduli. d: $p < 0.0001$ for control ECM moduli as compared to control PCM moduli. PCM moduli were unaffected by C-ABC digestion ($p = 0.68$) (N = 6 pigs, n = 24 regions per treatment). Hyal – e: $p < 0.00005$ for control ECM moduli as compared to Hyal digested ECM moduli. f: $p < 0.005$ for ECM moduli as compared to respective PCM moduli. PCM moduli were unaffected by Hyal digestion ($p = 0.43$) (N = 6 pigs, n = 24 regions per treatment). Elastase – g: $p < 0.000001$ for control ECM moduli as compared to elastase digested ECM moduli. h: $p < 0.00005$ for control ECM moduli as compared to control PCM moduli. i: $p < 0.05$ for control PCM moduli as compared to elastase digested PCM moduli (N = 3 pigs, n = 12 regions per treatment).

Figure 4.

Representative PCM scan regions for control and ADAMTS-4 digested sections. (A, B) Immunofluorescence labeling illustrated the distribution of type VI collagen around cell-sized voids. Scale bar = 5 μm. (C, D) Contour maps of calculated elastic moduli of the PCM scan regions shown. To highlight differences with digestion, contour maps are plotted on the same color scale. (E) Modulus progression from the PCM outer edge to the ECM in undigested control (black) and ADAMTS-4 digested (white) cartilage sections. *: $p < 0.05$ for control ECM as compared to digested ECM. Undigested control moduli demonstrated a trend toward an effect of distance ($p = 0.07$). ADAMTS-4 digested moduli demonstrated no variation with distance ($p = 0.48$). Moduli presented as mean $\pm$ standard error (N = 3 pigs, n = 9 regions per treatment).

Figure 5.

Representative PCM scan regions for control and C-ABC digested sections. (A, B) Immunofluorescence labeling illustrated the distribution of type VI collagen around cell-sized voids. Scale bar = 5 μm. (C, D) Contour maps of calculated elastic moduli of the PCM scan regions shown. To highlight differences with digestion, contour maps are plotted on the same color scale. (E) Modulus progression from the PCM outer edge to the ECM in undigested control (black) and C-ABC digested (white) cartilage sections. a: $p < 0.05$ for control PCM moduli as compared to control regions greater than 1.0 μm from the PCM outer edge. *: $p < 0.05$ for control moduli as compared to C-ABC digested moduli at each radial increment. #: Control moduli reached ECM-like values at the PCM outer edge. C-ABC digested moduli demonstrated no variation with distance ($p = 0.93$). Moduli presented as mean $\pm$ standard error (N = 6 pigs, n = 24 regions per treatment).

Figure 6.

Representative PCM scan regions for control and Hyal digested sections. (A, B) Immunofluorescence labeling illustrated the distribution of type VI collagen around cell-sized voids. Scale bar = 5 μm. (C, D) Contour maps of calculated elastic moduli of the PCM scan regions shown. To highlight differences with digestion, contour maps are plotted on the same color scale. (E) Modulus progression from the PCM outer edge to the ECM in undigested control (black) and Hyal digested (white) cartilage sections. a: $p < 0.05$ for control PCM moduli as compared to all control regions outside the PCM. b: $p < 0.05$ for Hyal digested PCM as compared to all digested regions outside the PCM. *: $p < 0.05$ for control moduli as compared to Hyal digested moduli at each radial increment. #: Control moduli reached ECM-like values 2.0 μm from the PCM outer edge. &: Hyal digested moduli reached ECM-like values 1.0 μm from the PCM outer edge. Moduli presented as mean $\pm$ standard error (N = 6 pigs, n = 24 regions per treatment).

Figure 7.

Representative PCM scan regions for control and elastase digested sections. (A, B) Immunofluorescence labeling illustrated the distribution of type VI collagen around cell-sized voids. Scale bar = 5 μm. (C, D) Contour maps of calculated elastic moduli of the PCM scan regions shown. To highlight differences with digestion, contour maps are plotted on the same color scale. (E) Modulus progression from the PCM outer edge to the ECM in undigested control (black) and elastase digested (white) cartilage sections. a: $p < 0.05$ for control PCM moduli as compared to control regions greater than 1.0 μm from the PCM outer edge. *: $p < 0.05$ for control moduli as compared to elastase digested moduli at each radial increment. #: Control moduli reached ECM-like values 1.5 μm from the PCM outer edge. Elastase digested moduli demonstrated no variation with distance ($p = 0.32$). Moduli presented as mean $\pm$ standard error (N = 3 pigs, n = 12 regions per treatment).