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Depth-Dependent Profiles of Glycosaminoglycans in Articular Cartilage by μ MRI and Histochemistry

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Abstract

Purpose: To quantify the distribution profile of glycosaminoglycans (GAGs) in articular cartilage with the magnetic resonance imaging (MRI) gadolinium (Gd) contrast method (the dGEMRIC procedure in clinical MRI) and correlate with histochemical results.

Materials and Methods: Fresh canine cartilage from seven humeral heads was harvested. Sixteen cartilage specimens were imaged at 13 μ m pixel resolution using the μ MRI T1-Gd method to generate 2D GAG maps in cartilage. Nineteen cartilage specimens from adjacent locations on the same joints were papain-digested to quantify the bulk GAG content in tissue. In addition, six cartilage specimens were microtomed into 40- μ m serial sections that were parallel with the articular surface. These sections were biochemically analyzed individually to determine the depth-dependent profiles of GAG concentration.

Results: The GAG concentrations between the μ MRI measurement and the bulk biochemical method have statistically significant agreement. The depth-dependent GAG profiles from the histochemical method (40 μ m depth resolution) have similar line shapes as that determined by μ MRI at 13 μ m resolution.

Conclusion: The GAG concentration as measured by μ MRI T1-Gd contrast method provides an accurate account of the macromolecular content in articular cartilage.

Keywords

cartilage; microscopic imaging; glycosaminoglycan; MRI; histochemistry; dGEMRIC

ARTICULAR CARTILAGE is a thin layer of connective tissue covering the ends of bones in joints. Even though the tissue is thin (typically 0.5–2 mm), articular cartilage has a unique depth-dependent structure, both biochemically (eg, concentration variations) and morphologically (eg, subtissue structural zones) (1–4). This depth-dependent organization of cartilage essentially defines its properties as measured by any technique in laboratories and hospitals. The ability to resolve the tissue's depth-dependent structure will therefore add to the comprehension of the various mechanisms that govern the natural functions of the tissue and signal the onset of tissue degradation. In recent years many studies have documented the

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depth-dependent properties of articular cartilage by biochemical (2,5–8), physical and optical (9–17), and mechanical means (5,18–23).

Proteoglycans are one of the three major molecular components in articular cartilage (the other two being water and collagen) and have a bottle-brush-like structure with a central protein core and the sidechains of glycosaminoglycan (GAG). The heavily sulfated GAG molecules carry a high concentration of negative charges and are closely packed in the tissue, thereby generating an osmotic pressure that contributes to the stiffness of articular cartilage as a load-bearing material. The reduction of GAG in the tissue will result in a biochemically and biomechanically weakened cartilage, eventually leading to clinical diseases such as osteoarthritis (OA) and other joint diseases. Since early degradations of the tissue usually occur localized at certain depths (24–26), it is critically important to measure not only the bulk GAG concentrations but also the GAG profiles across the tissue depth in cartilage.

There are several approaches to obtain a depth-dependent profile of GAG in articular cartilage, mainly 1) biochemical digestion, 2) light microscopy of stained sections, and 3) magnetic resonance imaging (MRI). The biochemical approach can provide an accurate account of the total GAG content in cartilage as a bulk quantity. This destructive digestion method can also be made into a ‘low-resolution imaging’ mode if one slices a tissue block into multiple sections that are parallel with the articular surface and analyze the bulk GAG content in each thin section (1,3,5,6,20,27,28). The light microscopy approach uses thin tissue sections that are purposely stained. If the staining protocol is specific to GAG, it is possible to obtain the molecular concentration from the intensity of the stained image (6,8). There is also a new method in the light microscopy approach that uses fluorescence immunolabeling to identify the chondrocytes under compression (18,23). The MRI approach, which is totally nondestructive, is known in clinical MRI literature as the dGEMRIC procedure (delayed gadolinium enhanced magnetic resonance imaging of cartilage). Based on the assumption that charged mobile ions will distribute in cartilage in an inverse relation to the concentration of the negatively charged GAG molecules, this MRI technique constructs the T1 images before and after the patient is injected with a charged MRI contrast agent, $\text{Gd}(\text{DTPA})^{2-}$ (10,11,13–16). Since Gd^{3+} is a paramagnetic ion that can shorten the T1 relaxation significantly and a low quantity of $\text{Gd}(\text{DTPA})^{2-}$ is usually harmless to humans, the dGEMRIC protocol has become an important clinical procedure in the detection and management of joint diseases.

This study concerns the quantitative determination of the GAG concentration profile as measured by the in vitro version of the dGEMRIC protocol, in which an intact block of tissue is soaked directly in the $\text{Gd}(\text{DTPA})^{2-}$ solution between the before/after imaging experiments. The microscopic resolution GAG profiles from μMRI were correlated with the GAG contents and profiles from the histochemical analysis. We hypothesized that the MRI T1-Gd approach can provide an accurate assessment of the GAG concentration profiles in cartilage nondestructively.

MATERIALS AND METHODS

Tissue Specimens

Fresh humeral heads were harvested within 3 hours of the animal death from canines that were sacrificed for unrelated cardiovascular experiments. The dogs were ≈ 1 –2 years old and musculoskeletally healthy. Rectangular blocks of tissue with full thickness of cartilage still attached to the underlying bone were cut from the central load-bearing region of humeral heads. Each specimen, which had dimensions of about $2 \times 2 \times 10$ mm, was bathed in saline and sealed in a precision glass tube. The results in this report, which came from 41 specimens harvested from the same area of the humeral surfaces from 7 dogs, were obtained with the same parameters and conditions. Among the specimens, which were adjacent to each other, 16 were used for μ MRI experiments and the rest for biochemical experiments.

Microscopic MRI (μ MRI) Determination of GAG

Several series of NMR spectroscopy experiments were performed to determine the relaxivity of $\text{Gd}(\text{DTPA})^{2-}$ in solutions of skim-milk powder at the concentrations of 0%–40%. As discussed in several reports in literature (13,33), these concentrations cover the approximate range of the macromolecular contents in most biological tissues (the solid content in cartilage is about 20%–35% (34,35)). R-values of $4.2 \text{ (mM sec)}^{-1}$ was found in our experiment for saline at 7T and 25°C, which agrees with the relaxivity values found in literature (14,29,30,33).

The μ MRI T1-GAG imaging followed the well-documented dGEMRIC procedure in the literature (10,11,29–31). Briefly summarized, each cartilage block was T1-imaged before ($T1_{\text{before}}$) and after ($T1_{\text{after}}$) a 10-hour immersion in 1 mM solution of a commercially available $\text{Gd}(\text{DTPA})^{2-}$ contrast agent (Magnevist, Berlex, NJ) at the room temperature ([H11015]25°C). The T1 images were converted to the $\text{Gd}(\text{DTPA})^{2-}$ concentration image of cartilage and subsequently to the GAG concentration image of cartilage (30).

The μ MRI experiments were performed using a Bruker AVANCE II MRI console interfaced to a 7T/89 mm superconducting magnet, commercial microimaging accessory, and a home-built 4-mm solenoid coil. The tissue block was placed in the magnet at the magic angle, which minimizes the influence of the dipolar interactions (32). The echo time (TE) of the imaging sequence was 8.6 msec and the repetition time (TR) of the imaging experiment was 1.5 and 0.5 seconds for the before- and after-soaking experiments, respectively. The 1-mm-thick imaging slice was transversely located in the middle of the 10-mm-long specimen. The 2D in-plane pixel size was 13 μm . The measurement of 2D T1 images used the inversion-recovery pulse sequence with five inversion points (for the $T1_{\text{before}}$, they were 0, 0.4, 1.1, 2.2, 4.0 sec; for the $T1_{\text{after}}$, they were 0, 0.1, 0.3, 0.5, 1 sec), which allowed the calculation of T1 relaxation in the tissue through a single exponential equation on a pixel-by-pixel basis. (To save time, the repetition time TR in μ MRI was less than $5T1$. In this case, we used a modified T1 fitting function, $Y = A(1 - B \exp(-t/T1))$, where B is less than 2 when TR [H11021] $5T1$.)

Biochemical Determination of GAG

The specimens for biochemistry experiments, which were harvested from adjacent locations on the same joints used in μ MRI specimens, were analyzed in two ways, parallel section analysis and bulk analysis. For parallel sections, a fresh specimen was frozen in water and sliced into a series of 40- μ m parallel sections using a cryotome (Reichert HistoStat Cryotome). These sequenced thin sections were individually dried in the oven at 70°C for 48 hours, weighed using a Cahn-2000 Micro Balance, and papain-digested in a 1:10 ratio at 67°C in an oven for 18 hours. For bulk analysis the whole-thickness fresh tissue was separated from the underlining bone and papain-digested immediately using the same approach. The digested cartilage extract was clarified by using 0.22 μ m cellulose acetate filter to remove any suspended particles (eg, residuals of bone) and a Millipore filter used for histochemical analysis. The total volume of the clarified extract was measured.

Aliquot volumes of 5–100 μ L from the test samples were taken for the glycosaminoglycan measurement spectrophotometrically by a modified DMMB (1,9-dimethylmethylene blue) protocol as follows. A Blyscan dye reagent (BioColor, Northern Ireland) containing an inorganic buffer with suitable surfactants and stabilizers was formulated for specific binding to GAGs. Absorbance values were measured at 656 nm using a Shimadzu UV-1700 spectrophotometer. Standard curves for the GAG analysis were generated by assaying known concentrations of Chondroitin 4-Sulfate, a GAG standard obtained from bovine trachea (BioColor). Separate standard calibration curves were generated for every individual measurement. The GAG content in the aliquot taken for analysis was then used to obtain the total GAG concentration present in the specimen and is expressed as μ g/mg or mg/ml wet/dry weights of the tissue.

RESULTS

μ MRI Determination of GAG

Cartilage imaging experiments using multiple concentrations of $\text{Gd}(\text{DTPA})^{2-}$ solution were performed. Despite the difference in $\text{Gd}(\text{DTPA})^{2-}$ concentrations (hence the difference in the $T1_{\text{after}}$ values), the calculated GAG concentration in cartilage is independent of the $\text{Gd}(\text{DTPA})^{2-}$ concentrations. The μ MRI results in this report came from 16 specimens that were soaked in 1 mM $\text{Gd}(\text{DTPA})^{2-}$ solution. Figure 1 shows a set of quantitative images from one representative cartilage-bone specimen. Although there is no $T1$ anisotropy in normal (healthy) cartilage (32,36), the existence of the magic angle effect in cartilage due to the inevitable $T2$ weighting in imaging influences the signal intensity. The $T1$ imaging experiments were therefore carried out at the magic angle ($\approx 55^\circ$), and the $T1$ images in Fig. 1a,b were rotated in the computer to the 0° orientation to simplify the data extraction. Since the paramagnetic Gd^{3+} ions shorten the $T1$ relaxation, the difference between the $T1_{\text{before}}$ and $T1_{\text{after}}$ can be used to calculate the $\text{Gd}(\text{DTPA})^{2-}$ concentration in the tissue (Fig. 1c), which can subsequently be converted to the GAG concentration (Fig. 1d) via the ideal electrochemical equilibrium theory (30).

Since there was no obvious topographical variation at the direction that was parallel with the articular surface within any single specimen (37), a rectangular region of interest (10 pixel

width) was selected from the middle region of each tissue image. At each pixel depth (13 μm) an averaged number was obtained from the 1D array of 10 pixels. The 1D profiles of the tissue properties as a function of the tissue depth therefore facilitate the quantitative evaluation. Figure 2 shows the averaged profiles from three independent specimens. Three features were observed. First, the error bars for the $T1_{\text{before}}$ and $T1_{\text{after}}$ measurement are small, which indicates the consistency of our quantitative $T1$ measurement and the small GAG variation among the specimens that were from different animals. (Reproducibility measurement on the same specimen was also carried out for a number of specimens; the reproducibility in our experiment was excellent.) Second, the concentration of the Gd(DTPA)^{2-} in the tissue decreases monotonically as the function of tissue depth. Finally, the concentration of the GAG in cartilage is approximately a linear function, increasing from the superficial zone to the radial zone.

Biochemical Determination of Bulk GAG

Nineteen fresh specimens from seven animals were digested to determine the bulk GAG concentration in cartilage. Table 1 compares the GAG concentration as determined by the bulk biochemical method and the μMRI method. Despite the inevitable influence of specimen-site/subject variations, a good agreement was found between the results from these two methods, which indicates that the GAG concentration as measured by MRI method is an accurate account of the GAG content in cartilage tissue.

Biochemical Determination of GAG in Parallel Sections

In addition to the bulk GAG measurement, six specimens (each was adjacent to a specimen used in the bulk measurement) were microtomed into 40- μm -thick serial sections that were parallel to the articular surface, where the depth-dependent bulk values could mimic the quantitative GAG profiles from MRI. Table 1 contains the summation of the bulk GAG concentrations from all parallel sections, which is about 46% lower than the GAG concentrations from the bulk specimens from their adjacent locations. Table 2 contains the detailed biochemical data from one specimen as well as the averaged GAG concentration from all six specimens. (In the calculation of GAG concentration the water concentration in cartilage was assumed to vary from 75% near the surface to 65% near the bone, and the density of cartilage was assumed to be 1.12 g/ml (38)). Figure 3 illustrates the depth-dependent profiles of tissue's dry weight and GAG concentration from one specimen. It is interesting to note that while the dry weight of the tissue sections is not a linear function of the tissue depth, the GAG concentration in the tissue varies approximately linearly as a function of the tissue depth (the correlation coefficient of a linear fitting is 0.964).

Correlation Between the μMRI Method and Histochemical Method

Figure 4 compares the averaged GAG profiles as determined by the μMRI method and the 'histochemical imaging' method using the parallel sections. As one can see, the shapes of the GAG profiles from these two methods were largely consistent with each other. However, the average measured values from the biochemical 'imaging' were about 46% of the corresponding values from the MRI method. Since the data from MRI is in statistical agreement with the biochemical data using the bulk specimens from the same joints, it is

clear that a portion of the tissue GAG has been lost during the process of analyzing the thin parallel sections (see Discussion).

DISCUSSION

This study demonstrates that the GAG concentration as determined by MRI T1-Gd contrast method using the cartilage-bone blocks is an accurate determination of the true GAG content in cartilage tissue (Table 1). From the available literature and the best of our knowledge, this is the first quantitative microimaging study that directly correlates the distribution profiles of GAG content in cartilage from these two distinctly different but important biomedical techniques.

Despite the fact that the biochemical data from the bulk specimens correlates well with the μ MRI data, the summation of the spatially resolved GAG profiles from the biochemical method of the parallel sections, however, generates a measured ‘bulk’ value that is lower than the true bulk experiments. Since the specimens in the bulk and parallel specimens were from the adjacent locations of the same joints, it is clear that there is a GAG loss during the process of analyzing the parallel sections, which could have several possible sources.

First, several reports in the literature have documented the histochemical analysis of parallel sections in cartilage study. The thickness of the sections in these reports varies from 70–300 μ m (1,3,5,6,20,27,28). Our section thickness of 40 μ m is probably the thinnest section thickness ever reported in the literature. A thinner section results in a better ‘resolution’ in histochemical ‘imaging’; however, a thinner section, where each section needs to be analyzed individually, introduces a larger experimental error (a random error). Second, the bulk specimens were enzyme-digested immediately after a minimal time delay and with no additional process, while obtaining and analyzing the parallel sections requires the specimens to be frozen for embedding, with a number of additional processes. In particular, some trace amount of GAG could be measured in the solution where the specimen was immersed. The amount of the GAG lost in the immersion solution depends on several experimental factors, including the temperature and duration of the immersion period, and the type of the solution. This will introduce another experimental error in the GAG determination by histochemistry, a systematic error. Third, the method of measuring tissue’s wet weight was different between the bulk specimens and parallel sections. For bulk specimens the fresh tissue blocks were directly weighed using a microbalance. For parallel sections, getting a reliable measurement of the wet-weight of any thin section was nearly impossible with our equipment—the 40- μ m thin section gets dehydrated quickly and constantly while it is being weighed. For this reason, the thin sections were dried in the oven to obtain its dry weight, which was later converted into wet weight based on the assumption of $\approx 70\%$ water concentration in articular cartilage, which could introduce another systematic error. Finally, when one microtomes the parallel sections, it is often difficult to know where is the start of the tissue block’s surface, which can result in a lower dry weight for the first several sections (Table 2). In addition, any surface curvature of a tissue block further contributes to the uncertainty of the dry tissue amount for the first several sections. For these reasons, the percentage errors in the GAG concentration were highest for the first several

sections and were lowest for the deepest sections. This large error near the surface region might explain the difference in the GAG quantity between the two GAG profiles in Figure 4.

Although the physical principle of this in vitro T1-Gd contrast method is identical to that of the in vivo dGEMRIC protocol, the mechanisms that transport the gadolinium ions into the tissue are quite different. In the in vitro case (this report), the small tissue blocks are soaked directly in ample volume of Gd(DTPA)^{2-} solution where self-diffusion is probably the only mechanism responsible for the ion transportation into the tissue. In the in vivo case (dGEMRIC), the gadolinium ions are injected into either a vein or the joint cavity where a much more complicated set of mechanisms are involved in the ion transportation. There are several complications in the transportation of the gadolinium ions in the in vivo dGEMRIC procedure, such as the diffusion limitation of gadolinium ions into cartilage and the deviation of a true R-value due to the changes in the solid concentration in a lesioned tissue. These complications would result in a less accurate correlation between the gold-standard measurement (biochemistry) and the clinical imaging. The fact that the summation of the spatially resolved GAG profiles by μMRI experiments is in statistical agreement with the bulk GAG determination by the classical biochemical method offers an assurance that the physical mechanisms in the dGEMRIC method can serve as a truthful tool to quantitatively monitor the tissue's GAG content, which is considered to be responsible for the mechanical strength of articular cartilage as a load-bearing material.

The high sensitivity to GAG content using the in vitro T1-Gd contrast method has two implications. First, since the μMRI T1-Gd method has high spatial resolution and can map the GAG concentration in cartilage with high sensitivity nondestructively, this work validates the potential of the high-resolution imaging procedure, which can be directly applied to monitoring of cartilage development, degeneration, and repair in vitro in laboratories. Second, the in vitro validation in this report lends strong indirect support to the clinical potential of the in vivo dGEMRIC procedure since the same physical mechanism is used in both environments. Although a less direct correlation could be assumed between the gold-standard measurement (biochemistry) and the clinical imaging due to the practical complication discussed above, the unique value of clinical detection of early osteoarthritic lesion relies on the visualization and comparison of cartilage in a whole joint nondestructively. Comparing a local tissue with its surrounding tissue is an equally invaluable approach that offers complementary information to the detailed knowledge of the actual GAG content in cartilage, which does vary among individual animals and humans.

In conclusion, this study demonstrated that the GAG concentration as measured by μMRI is an accurate account of the macromolecular content in articular cartilage. To the best of our knowledge, this is the first study in cartilage research that correlates directly the GAG distribution profiles between the gold standard in histochemistry and the noninvasive MRI at microscopic resolutions. By analyzing parallel sections of cartilage, this work demonstrates that the GAG concentration profile in articular cartilage varies as a function of the tissue depth approximately linearly in cartilage.

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Figure 1.

T1 images of cartilage before (a) and after (b) the specimens were immersed in 1 mM Gd(DTPA)²⁻ solution. c: The image of gadolinium concentration in cartilage. d: The image of the GAG concentration in cartilage.

Figure 2.
a: T1 profiles before and after the $\text{Gd}(\text{DTPA})^{2-}$ immersion and **(b)** the concentration profiles of gadolinium and GAG in articular cartilage.

Figure 3.

The profiles of biochemical details of the 40-μm parallel sections microtomed from a single cartilage section. Although the dry weight of the tissue is not a linear function of the tissue depth, the GAG concentration varies approximately linearly.

Figure 4.

The correlation of the quantitative GAG profiles from MRI and biochemistry. The difference of the total quantity could be from the additional process in preparation of the thin sections. See text for discussion.

Table 1
Bulk GAG Concentrations in Cartilage as Determined by the Histochemical and μ MRI Methods

	GAG Concentration (mg/ml, w.w.)	Sample No.	Statistics
Summation of μ MR imaging profiles	69.1 $\pm$ 6.4	16	]
Biochemistry of bulk specimens	64.9 $\pm$ 6.8	19	
Summation of biochemistry of 40- μ m parallel sections	36.2 $\pm$ 9.6	6	

w.w. = wet weight.

* The correlation between these two groups of data was analyzed using the nonparametric Wilcoxon-Mann-Whitney test in commercial software, KaleidaGraph 4.0 (Synergy Software, Reading, PA). The P -value of the test was 0.076, which indicates that there is no statistically significant difference between the medians of these two groups.

Table 2Histochemical Results From 40- μ m Parallel Sections

Depth (μ m)	Details of One Specimen			GAG Concentration of Six Specimens		
	Tissue Weight (μ g, d.w.)	Total GAG (μ g)	GAG Concentration (mg/ml) (d.w.)	GAG Concentration (mg/ml) (w.w.)	Mean	Standard Deviation (mg/ml, w.w.)
20	10	0.201	22.45	5.61	6.79	5.21
60	23	0.875	42.59	10.94	7.89	4.58
100	35	1.551	49.63	13.08	16.03	7.95
140	41	2.389	65.27	17.65	18.27	7.71
180	47	2.085	49.67	13.77	21.72	8.60
220	39	2.471	70.95	20.15	23.97	10.15
260	56	4.811	96.22	27.98	27.64	13.98
300	50	3.608	80.83	24.05	33.49	18.66
340	55	4.361	88.81	27.04	36.36	19.46
380	50	5.473	122.59	38.15	41.89	14.50
420	55	5.770	117.50	37.37	45.71	13.72
460	55	6.664	135.70	44.08	53.82	11.01
500	57	7.574	148.83	49.35	55.56	7.27
540	66	6.762	114.74	38.83	48.10	8.73
580	60	7.558	141.08	48.70	46.15	15.15
Total	699	62.15				
Mean			89.79	27.78		

d.w. = dry weight; w.w. = wet weight.