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Induction of Advanced Glycation End Products and Alterations of the Tensile Properties of Articular Cartilage

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Objective. **To determine whether increasing advanced glycation end products (AGEs) in bovine articular cartilage to levels present in aged human cartilage modulates the tensile biomechanical properties of the tissue.**

Methods. **Adult bovine articular cartilage samples were incubated in a buffer solution with ribose to induce the formation of AGEs or in a control solution. Portions of cartilage samples were assayed for biochemical indices of AGEs and tested to assess their tensile biomechanical properties, including stiffness, strength, and elongation at failure.**

Results. **Ribose treatment of cartilage induced increases in tissue fluorescence, absorbance, and pentosidine content ($P < 0.001$ for each comparison) by amounts similar to those that occur during aging in humans. Ribose treatment of cartilage also induced an increase in dynamic modulus (60% increase) and strength (35% increase), and a decrease (25% decrease) in strain ($P < 0.001$ for each comparison).**

Conclusion. **The concomitant increase in AGEs and alteration of tensile properties of cartilage after ribose treatment suggest that aging-associated changes in AGEs have functional consequences for this tissue. The AGE-associated increases in strength and stiffness of cartilage may be beneficial by counteracting the decreases in these properties that are associated with degeneration. Conversely, the AGE-associated decrease in failure length, or increase in brittleness, together with increased stiffness may predispose cartilage to increased stress concentration, fracture, and aging-associated biomechanical dysfunction.**

Articular cartilage normally functions as a wear-resistant, low-friction, load-bearing material in diarthrodial joints (1). The load-bearing biomechanical function of cartilage is governed in large part by the balance between the swelling propensity of proteoglycan molecules and the restraining function of the collagen network (2). With advancing age in adult humans, there is an increase in the prevalence of osteoarthritic cartilage degeneration (3). In osteoarthritic cartilage, the restraining function of the collagen network is deficient (4). However, it is unclear if aging-associated alterations of cartilage result in biomechanical changes that predispose this tissue to degeneration and osteoarthritis.

With aging, there is a decrease in the biomechanical function of the collagen network of macroscopically normal human articular cartilage. The tensile strength of articular cartilage reflects primarily the properties of the collagen network of the tissue, as does tensile stiffness, when measured at a relatively high level of stress or strain (1,5). In macroscopically normal human articular cartilage, from age 20 years to 90 years, the tensile strength was found to decrease 4-fold in knee tissue (6) and 2–6-fold in hip tissue (7,8), with tensile stiffness generally exhibiting the same trends (6,7). These tensile properties appear to be especially sensitive to age-associated changes, in contrast to the confined compression modulus and permeability of cartilage (9).

With aging, one of the major biochemical changes in articular cartilage is the accumulation of advanced glycation end products (AGEs), resulting from the spontaneous reaction of reducing sugars with pro-

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teins (10). The initial step in this reaction is the formation of a Schiff base through the condensation of a sugar aldehyde with an ε-amino group of (hydroxy)lysine or arginine residues in proteins. After a cascade of largely unknown reactions, the Schiff bases are ultimately converted into a heterogeneous collection of chromophores and fluorophores, collectively known as AGEs.

Since most of these AGEs have not yet been isolated or characterized, a few well-characterized AGEs are routinely used as markers for the process of nonenzymatic glycation. One such marker is pentosidine, a fluorescent AGE formed between lysine and arginine residues (11). More general measures for AGE are tissue browning, fluorescence, and modification of the glycation-sensitive amino acids arginine, lysine, and hydroxylysine (12). Once formed, AGEs appear to be removed from the body only when the protein involved is removed. Consequently, AGEs accumulate, especially in those proteins with slow turnover such as articular cartilage collagen (13), although all proteins are prone to AGE formation. From age 20 onward (skeletal maturity), levels of AGEs increase linearly with age in articular cartilage (13).

Accumulation of AGEs in articular cartilage results in alteration of certain biomechanical functions of the collagen network (11). Increasing levels of AGEs, due to either natural variations in vivo or treatment with reducing sugars (e.g., ribose or threose) in vitro, led to a decrease in the "instantaneous deformation" of cartilage in unconfined compression (11). While "instantaneous deformation" is taken to be an index of the stiffness of the collagen network (11,14,15), it remains to be determined whether more direct measures and other properties of the collagen network are also affected by AGE accumulation, and how such changes may relate to aging-associated changes in cartilage mechanical properties.

The objective of the present study was to test the hypothesis that AGE formation modulates the biomechanical properties of the collagen network of articular cartilage. We examined the effect of ribose treatment on cartilage tensile stiffness, strength, and failure strain.

MATERIALS AND METHODS

Materials used for cartilage isolation and treatment, biochemical analysis, and biomechanical testing were obtained as described previously (12,16–20). In addition, black 96-well plates were obtained from Fisher Scientific (Tustin, CA).

Cartilage isolation and treatment. Cartilage was isolated from a total of 45 osteochondral blocks (10 × 5–mm cartilage area) harvested from the patellofemoral grooves of 3 macroscopically normal adult bovine knees (Haedrich's Tip Top Meats, Carlsbad, CA), essentially as described previously (19). The long axes of the blocks were in the medial–lateral direction and, thus, approximately parallel to the split line direction. Each block was placed in a microtome, and four 0.25 mm–thick cartilage sections were sequentially cut. These sections were then cut into rectangular pieces with an area of 10 × 3–mm. The pieces were identified as layers I, II, III, and IV, with layer I including the intact articular surface.

The cartilage samples were distributed between 2 treatment groups of approximately the same number of samples from layers I, II, III, and IV. The treatment conditions were adopted from previous studies (11), as follows. Sham-treated samples were maintained in 0.15*M* NaCl with 50 m*M* potassium phosphate buffer containing 25 m*M* EDTA, 25 m*M* ε-amino-*n*-hexanoic acid, 5 m*M* benzamidine HCl, and 10 m*M* *N*-ethylmaleimide at pH 7.2 for 86 hours at 37°C. Ribose-treated samples (to induce AGE formation) were maintained in the same solution as the sham-treated samples, but with the addition of 0.6*M* ribose. After treatment, sections were rinsed 3 times in 0.15*M* NaCl at 4°C over 24 hours.

Since AGE accumulation is known to result in a yellow/brownish color of cartilage, the color of the cartilage samples following sham or ribose treatment was noted. From each cartilage sample, a tapered strip (6) with a gage region of 4 × 0.80 mm (length × width) was prepared for tensile biomechanical testing. The remaining tissue adjacent to each tensile strip was saved for biochemical analysis.

Biochemical analysis. The tissue adjacent to each tensile strip was weighed while wet and solubilized with proteinase K (20). Portions of the digest were assayed for hydroxyproline, as a measure of collagen (21), and normalized to the wet weight. Other portions of the digest were analyzed for fluorescence at excitation and emission wavelengths of 335 nm and 395 nm, respectively, which is 1 pair of excitation/emission wavelengths indicative of AGEs (22). The measurement was done by assaying a portion of each sample using a black 96-well plate (Fisher Scientific) in a Molecular Devices (Sunnyvale, CA) SpectraMax GeminiXS plate reader (photomultiplier tube setting low, 9-nm bandwidth, average of 6 readings per well), and the resultant fluorescence was normalized to the wet weight.

After biomechanical testing (see below), portions of the tested tensile strips were lyophilized and combined to generate a smaller number of pooled samples for more specific AGE analysis. In these studies, half of each of the tested strips from 3–4 samples were combined into sample pools, with each pool containing an approximately similar distribution of the layers (I–IV) of cartilage from an individual treatment group. Indices of cartilage AGE levels were determined as described previously (11,12,17). Briefly, cartilage samples were solubilized by digestion with papain. Portions of papain digests were analyzed for AGE-associated absorption at 340 nm and for fluorescence at excitation and emission wavelengths of 360 nm and 460 nm, respectively (another pair of excitation/emission wavelengths indicative of AGEs) (11). Other portions of papain digests were hydrolyzed in 6*N* HCl for 18 hours at 110°C and then analyzed by high-performance liquid chromatography for the AGE crosslink pentosidine (11,12,17) and amino acids to assess AGE-associated modification of the amino acids arginine, lysine, and hydroxylysine (12,16). The nonenzymatic glycation parameters, absorption and fluores-

cence, were expressed per amount of hydroxyproline, while the AGE measures, pentosidine and amino acid modification, were expressed per collagen triple helix (assuming 300 hydroxyproline residues per triple helix).

Biomechanical testing. The thickness of the gage region of each tensile strip was measured based on a marked decrease in electrical resistance at contact (with minimal deformation). This allowed calculation of the cross-sectional area of the gage region (i.e., ~0.25 × 0.80 mm = 0.2 mm^2). Each strip was then clamped in a mechanical tester (23) and elongated to failure at 5 mm/minute, as used extensively in previous studies (6,24), under continuous hydration with phosphate buffered saline. The load was measured at 1-second intervals. Three parameters were obtained from the load-displacement curve. Load and displacement were converted to stress (defined as the load normalized to the initial cross-sectional area of the gage region) and strain (defined as the elongation distance normalized to the initial gage length), and the dynamic modulus was calculated as the corresponding slope of the stress–strain curve from 10–50% of the maximum stress. Tensile strength was determined as the maximum stress. The failed portions of each tensile strip, resulting from the tensile test, in addition the adjacent cartilage samples obtained during preparation of the tensile strips, were also saved for biochemical analysis (described above).

Statistical analysis. Tissue fluorescence and biomechanical data were log-transformed (to improve the assumption of equal variance among the sample groups), and then analyzed by analysis of variance to assess the effects of ribose treatment and depth from the articular surface (as fixed factors) and to account for animal effects (as a random factor). The ribose-treated and sham-treated groups were compared as a planned test, while other comparisons were done with Tukey's post hoc test. For absorption, fluorescence, pentosidine, and amino acid data from pooled samples, the effect of ribose treatment was assessed by 2-tailed t-test, assuming unequal variances. Data are expressed as the mean ± SEM.

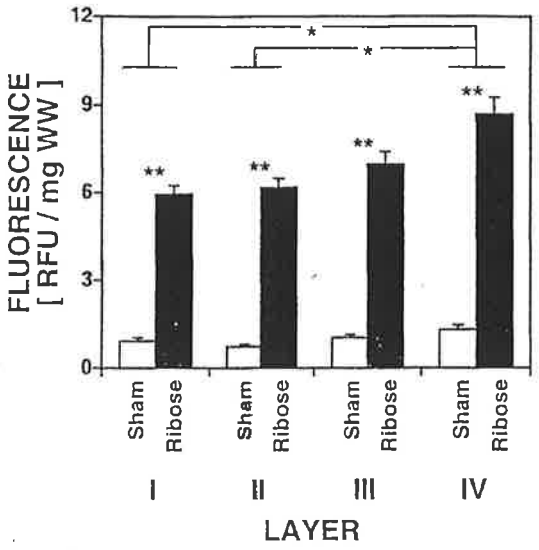

Figure 1. Sham-treated and ribose-treated cartilage adjacent to the strips tested for tensile properties were analyzed for advanced glycation end product (AGE)–associated fluorescence at an excitation wavelength of 335 nm and an emission wavelength of 395 nm (see Materials and Methods). Fluorescence (relative fluorescence units [RFU]) was normalized to the wet weight (WW) of the tissue. RFU/WW values were normalized so that sham-treated samples had an average value of 1; ribose-treated samples are relative to sham-treated samples. Ribose treatment increased the fluorescence over that in the sham-treated tissue, without interaction between the treatment and cartilage layer. The overall fluorescence increased with increasing depth from the articular surface. Values are the mean and SEM of 13–15 samples per treatment group for each cartilage layer (I–IV, where layer I is the articular surface). * = $P < 0.05$; ** = $P < 0.001$.

RESULTS

The cartilage color remained white in sham-treated samples, but became yellow-brown after ribose treatment. The hydroxyproline (collagen) content was similar in cartilage samples after sham treatment (11.6 ± 0.8 mg/gm wet weight) and ribose treatment (12.3 ± 0.3 mg/gm wet weight). Thus, the expression of all AGE parameters, normalized to hydroxyproline (or the calculated collagen) content, provided an index of the AGE content relative to the tissue wet weight as well as to collagen, and such normalized values were used in subsequent analyses to facilitate comparison with previous results (11,12).

Ribose treatment induced AGE formation, as indicated by a number of biochemical measures. The levels of general measures of AGEs were increased markedly by ribose treatment. AGE-associated fluorescence at an excitation wavelength of 335 nm and an emission wavelength of 395 nm of solubilized cartilage was increased up to 8.5-fold (Figure 1). Fluorescence was also dependent on cartilage depth from the articular surface ($P < 0.01$), but there was no interaction between depth and ribose treatment ($P = 0.95$). Thus, the fluorescence of sham-treated samples increased by 44% from layer I to IV, and the fluorescence of ribose-treated samples increased similarly, by 46%, from layer I to IV. Because of the similar and relatively small percentage increase with depth in this AGE parameter for both sham- and ribose-treated samples, other analyses of AGEs were done on samples pooled from different layers.

The other specific and general measures of AGEs were also increased significantly by ribose treatment (Table 1). The content of the chemically characterized AGE, pentosidine, was increased 131-fold. AGE-associated fluorescence at excitation 360 nm and emission 460 nm of solubilized cartilage was increased 12-fold. Absorption at 340 nm of solubilized cartilage was

Table 1. Biochemical indices of advanced glycation end products in sham-treated and ribose-treated cartilage

Biochemical index of advanced glycation end products	Sham-treated cartilage (n = 15)	Ribose-treated cartilage (n = 15)
Pentosidine (moles/mole of collagen)	0.67 ± 0.38	87.63 ± 4.36*
Fluorescence at 360/460 nm (per nmole of hydroxyproline)	0.87 ± 0.05	10.56 ± 0.51*
Absorption at 340 nm (per μmole of hydroxyproline)	0.035 ± 0.001	0.227 ± 0.008*
Arginine, lysine, and hydroxylysine (moles/mole of collagen)	274 ± 4	212 ± 3*

* $P < 0.001$ versus sham-treated cartilage.

increased 6.5-fold. The ribose-sensitive amino acids, arginine, lysine, and hydroxylysine, were decreased by 23%.

Ribose treatment also affected the tensile biomechanical properties of the cartilage samples. Each of the tensile properties was affected significantly by ribose treatment ($P < 0.01$ for each comparison). The slopes of the load-displacement curves were steeper for the ribose-treated cartilage than for the sham-treated cartilage (Figures 2A–D). Since the samples were cut to be similar and the measured thickness was only slightly higher in samples from layer I than from layers II–IV (mean ± SEM 0.286 ± 0.013 mm versus 0.251 ± 0.011 mm), the ribose-treated cartilage had a higher dynamic modulus than the sham-treated cartilage (average of 59% higher among all cartilage layers) (Figures 2E–H). Each of the samples fractured in the gage region, and the peak in load was greater for the ribose-treated cartilage than for the sham-treated cartilage, with a correspondingly higher strength (average of 35% higher) (Figures 2I–L). The peak in load occurred at a lesser elongation in ribose-treated cartilage than in sham-treated cartilage, with a correspondingly lower fracture strain (average of 25% lower) (Figures 2M–P).

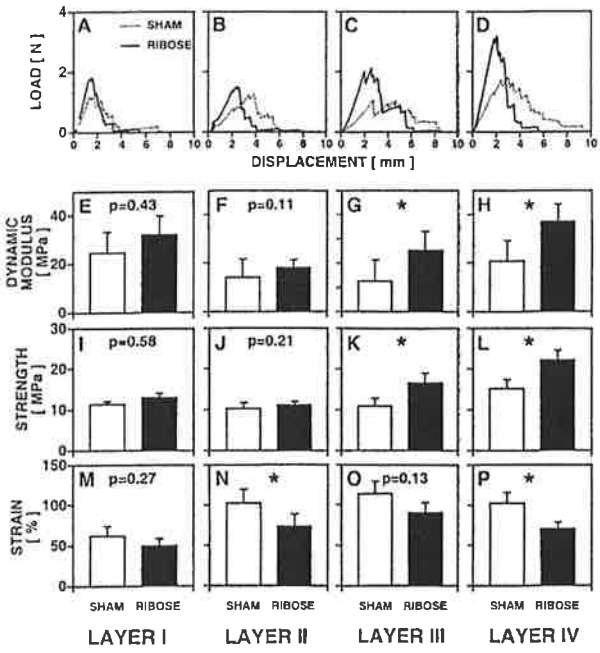

Figure 2. Effect of ribose treatment on the tensile properties of cartilage from sequentially cut layers (I–IV, where layer I is the articular surface). **A–D,** Average load-displacement profile of sham- and ribose-treated samples for each layer. **E–H,** Dynamic modulus, **I–L,** strength, and **M–P,** strain at failure for each cartilage layer. Values are the mean and SEM of 14–15 samples per experimental group. * = $P < 0.05$. Ribose treatment markedly increased the dynamic modulus and strength of the cartilage, while decreasing the strain required to induce failure.

The tensile properties were also dependent on the location of the tissue specimen with respect to the articular surface ($P < 0.001$ for each comparison), but without a statistically significant interaction between ribose treatment and layer effects ($P = 0.63$–0.82). The dynamic modulus and strength of cartilage generally decreased with increasing depth from the articular surface, from layer I to layer III (Figures 2E–G and I–K), while failure strain generally increased (Figures 2M–O). The deepest layer (layer IV) exhibited somewhat distinct properties, with dynamic modulus and strength being relatively high (Figures 2H and L), as was failure strain (Figure 2P). The effects of ribose treatment did not reach statistical significance in the most superficial layer (Figures 2E, I, and M), whereas the effects on the other layers were fairly consistent with the overall effect of ribose treatment (Figures 2F–H, J–L, and N–P).

DISCUSSION

The current study investigated the effect of ribose treatment of adult bovine articular cartilage on the

biomechanical function of the collagen network, as indicated by the tensile properties of the tissue. The ribose treatment procedure used in the present studies was used previously on young adult human cartilage to induce biochemical changes in AGEs, as indicated by pentosidine, to levels similar to those which occur during normal cartilage aging (11). The biochemical changes induced in bovine cartilage (Figure 1 and Table 1) were of the same magnitude as the changes found in human cartilage (11). In the same bovine cartilage samples, ribose treatment induced an increase in tensile strength and stiffness, and a decrease in failure strain (Figure 2). Taken together, these findings suggest that there are a number of specific biomechanical consequences of the naturally occurring age-associated increase in AGE levels in cartilage.

We used cartilage from the patellofemoral groove of adult bovines, and extrapolation of the results to changes in human cartilage during aging requires consideration of a number of factors. The bovine source of articular cartilage provides a broad and relatively flat joint surface over which multiple tensile tissue samples can be isolated, and it can be obtained from an adult animal, yet still be typically free of cartilage degeneration. The magnitude and depth-dependent variation in the tensile properties of the adult bovine cartilage tested in the present study are consistent with some, but not all, of the properties of adult human cartilage. The tensile stiffness and strength of adult bovine cartilage (14–27) (Figures 2E–L) are somewhat lower than the tensile stiffness and strength of adult human articular cartilage (5–8,28). In addition, the variation in tensile strength and stiffness with depth from the articular surface found in the present study is intermediate to the variation found in immature and older adult human cartilage (6–8,25,28,29). On the other hand, the failure strain (Figures 2M–P) is highly consistent both in magnitude and depth-variation with that found previously for articular cartilage from adult bovines (25) as well as cartilage from the lateral femoral condyle of young adult humans (29). Taken together, these considerations support the use of adult bovine cartilage tissue in the current investigation.

The AGE levels in cartilage samples (Table 1) were increased by ribose treatment from the low levels typical of that of immature human cartilage to levels comparable to those in human articular cartilage from individuals of an advanced age (11). Although pentosidine accounts for a small proportion of the total Maillard crosslinks, it serves as a sensitive and specific marker for the accumulation of nonenzymatic glycation products. The other absorption, fluorescence, and biochemical markers of AGEs in bovine cartilage (Figure 1 and Table 1) were also increased markedly by ribose treatment, as they were with normal or ribose-induced aging in previous studies (12,22).

The effects of ribose treatment on cartilage tensile properties are generally consistent with and extend the information available from previous studies on the biomechanical consequences of AGEs. Induction of AGEs has been associated with alteration of the biomechanical properties of a number of other collagenous tissues, such as lens capsule (30), rat tail tendon (31,32), skin (33), and the collagen network of bone (34). The ribose-induced changes in cartilage tensile properties found in the present study (Figure 2), including increased stiffness and strength as well as decreased elongation at failure, are all indications that AGEs modify the function of the collagen network of cartilage. The ribose-induced increase in tensile stiffness measured in our study is consistent with the 25% decrease in "instantaneous deformation" of cartilage in unconfined compression, a measure that is also influenced by the stiffness of the collagen network (11). It is difficult to compare these results in more detail because of differences in loading configuration, tissue source, and the extent of AGE induction, as noted above. The AGE-associated increase in cartilage strength (35%) and decrease in failure strain (25%) have not been noted previously. It appears that AGE induction causes changes in the compressive and shear properties of cartilage (35), as well as changes in the tensile properties we studied. It remains to be determined if AGE induction affects the viscoelastic behavior of cartilage in compression, shear, and tension by altering tissue properties such as elastic moduli or hydraulic permeability.

These biomechanical changes induced in cartilage by ribose treatment may be related to changes in adult human cartilage during aging or osteoarthritis. With advancing age and histopathologic features of osteoarthritis, the tensile strength and stiffness of human cartilage decreases while AGEs accumulate (6–8,36). These biomechanical changes are opposite to those in the current study that were induced by ribose treatment to form AGEs (Figure 2). Thus, it is unlikely that the formation of AGEs is directly responsible for these age-related changes in biomechanical properties. Indeed, the AGE-associated increase in strength and stiffness of cartilage may be beneficial in counteracting the decrease in these properties associated with degeneration and collagen degradation (14). Alternatively, AGE-associated changes in cartilage may have a para-

doxical effect on biomechanical function; the decrease in length at failure is characteristic of a material with increased brittleness, and this in combination with an increase in collagen network stiffness may predispose the cartilage to undergoing increased stress concentration, fracture, and ultimately, aging-associated biomechanical dysfunction (11).

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