Ultrasonic propagation properties of articular cartilage at 100 MHz

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(Received 14 December 1987; accepted for publication 18 November 1989)

A pilot study on articular cartilage assessed the contributions of individual matrix components to ultrasound propagation. The influence of collagen fibril orientation and collagen cross linking was also assessed. Sections of adult bovine articular cartilage cut both parallel and perpendicular to the articular surface were examined using the scanning laser acoustic microscope (SLAM) operating at an ultrasonic frequency of 100 MHz. A set of samples was evaluated that had been sequentially treated by enzymes to (1) remove 85% of the chondroitin sulfate; (2) remove remaining glycosaminoglycans, glycoproteins, and other noncollagen proteins, leaving only the collagen fibril network; and (3) disrupt the collagen intermolecular cross links. Two striking observations were made: a profound effect of the "preferred" collagen fibril orientation on ultrasonic speed and a marked increase in attenuation coefficient when intermolecular cross links were broken in the collagen.

PACS numbers: 43.80.Cs

INTRODUCTION

The propagation of ultrasound in biological tissues is poorly understood.[1] In connective tissues, for instance, it is not clear to what degree the collagen fibril framework and the embedded interfibrillar molecules (proteoglycans and matrix proteins) contribute to sound conduction. These considerations are of interest in the clinical use of ultrasound since the composition and configuration of connective tissues change during disease states (e.g., cirrhosis of the liver and degenerative joint disease) as well as physiologic processes (e.g., wound healing). Using articular cartilage, which is a relatively homogeneous, avascular connective tissue, we have begun to study this question by selectively degrading components of the extracellular matrix.

The collagen fibrils of articular cartilage are polymers largely of type II collagen molecules. At least 13 genetically distinct types of collagen molecule have been identified.[2] Types I, II, and III collagens are the common, fibril-forming types that form the main fabric of connective tissues. Type II (a molecule of chain composition $[\alpha 1\ (II)]_3$) is the form found in and peculiar to hyaline cartilages. Type IX collagen is a quantitatively minor collagen molecule that is found associated with type II collagen fibrils.

Ultrastructural studies of adult cartilage show that the preferential orientation of the collagen fibrils is quite different for the tangential zone near the articular surface (approximately 200 μm deep) compared to the intermediate (transitional) and deep (radial) zones (see Fig. 1). In the tangential zone, the collagen is more densely packed and oriented approximately parallel to the articular surface while in the deeper, radial zone, the collagen is more loosely packed and oriented approximately perpendicular to the articular surface.[3,4] In the transitional zone, the orientation is more random and, as the name suggests, represents a gradual transition from one preferred orientation to the other. Hence, several questions can be addressed using this cartilage model, including: the effect of collagen orientation on the ultrasonic propagation properties, and the effects of removing individual glycosaminoglycans (GAGs) and proteoglycans and destroying cross links in the collagen framework.

I. METHODS

A. Articular cartilage

Articular cartilage was obtained immediately postmortem from the stifle (knee) joint of 1- to 2-year-old adult steers. Full thickness cartilage plugs were excised from the patellar groove with a cylindrical 6-mm-diam punch and washed in saline plus protease inhibitors (2-mM phenylmethylsulfonyl fluoride, 10-mM N-ethyl maleimide, 2-mM

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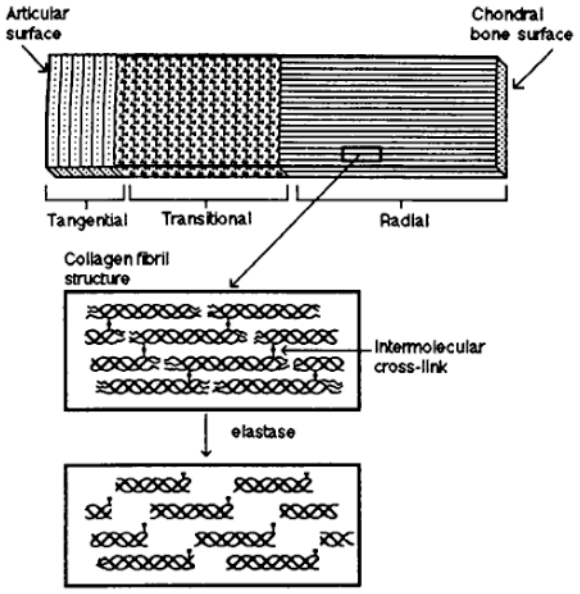

FIG. 1. Diagram of the predominant orientations of collagen fibrils in different zones of articular cartilage. In the surface 200 μm (tangential zone), fibrils lie primarily parallel to the articular surface, but their axes are randomly oriented in the plane of the articular surface. Fibrils are, in general, randomly oriented in the transitional zone and perpendicular to the articular surface in the deep, radial zone. The lower panels illustrate the head-to-tail positions of cross links between collagen molecules packed in fibrils as well as the subsequent disruption of cross links by the telopeptidase activity of pancreatic elastase.

ethylene diamine tetraacetic acid, and 5-mM benzamidine-HCl) prior to enzyme digestion. A sequential enzyme extraction procedure[5] was used to deplete cartilage plugs of specific matrix constituents. The enzymatic alteration included the following steps: (1) incubation of plugs in buffer alone (0.05-M Tris-HCl, 0.06-M Na acetate, *p*H 8.0) (control or *normal*); (2) removal of 85% of the chondroitin sulfate and dermatan sulfate using the enzyme chondroitinase ABC, which degrades the glycosaminoglycan chains of the proteoglycan to disaccharides (*CS-depleted*) [Chondroitinase ABC is the established biochemical name of a bacterial enzyme that degrades three forms of sulfated glycosaminoglycan: chondroitin-4-sulfate (chondroitin sulfate A), dermatan sulfate (chondroitin sulfate B), and chondroitin-6-sulfate (chondroitin sulfate C).]; (3) removal of the remainder of the noncollagenous matrix using streptomyces hyaluronidase and trypsin digestion (*collagen only*); and (4) functional disruption of the intermolecular crosslinks using porcine pancreatic elastase, a proteinase that cleaves collagen telopeptides (*cross-links cleaved*).[6] All plugs were washed with distilled, deionized water for 60 min at 4 °C to remove buffer salts and enzymes before mounting for acoustical measurements.

At the completion of the enzyme digestions, the treated cartilage plugs, along with the untreated plugs, were mounted on cork with standard mounting media for frozen sections Ames Tissue-Tek® O.C.T. [(optimal cutting temperature), a polyvinyl alcohol, benzalkonium chloride, and polyethylene glycol gel], frozen in liquid nitrogen, placed in Ziploc® bags and shipped on dry ice from the University of Washington to the Bioacoustics Research Laboratory at the University of Illinois where they were stored in a Revco freezer at −70 °C pending analysis by the scanning laser acoustic microscope (SLAM). The samples were sent in duplicate and coded so that the investigators making ultrasound measurements did not know how the cartilage samples were treated. For evaluation by the SLAM, each 6-mm disk of cartilage was cut in half (see Fig. 2). From one half, a 2-mm plug was bored for sectioning parallel to the articular surface. The other half was trimmed and mounted so that sections perpendicular to the articular surface could be cut.

It has been demonstrated that rapid freezing in liquid nitrogen does not affect the mechanical properties of skin and wound tissue.[7] Similarly, no differences were observed in ultrasonic impedance among several tissues studied before and after freezing.[8] It was necessary for the cartilage specimens to be frozen in the present study for both transporting specimens and sectioning them for the SLAM, but the study was designed so that all experiments could be accomplished with only 1 cycle of freeze–thaw.

Specimens of known thickness (25, 40, 50, and 60 μm for perpendicular samples and 25, 50, and 70 μm for parallel samples) were mounted on glass slides with albumin fixative so that the position of the articular surface was always known. A record of depth from the articular surface was kept for the parallel section specimens.

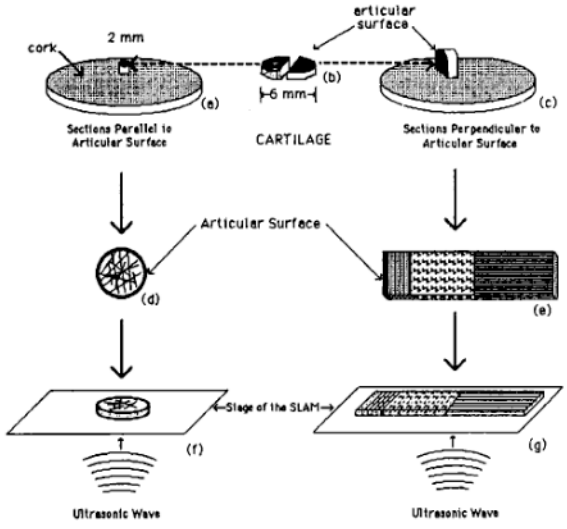

FIG. 2. Diagram of cartilage orientation for sectioning. The 6-mm-diam disks of cartilage with articular surface upwards were bisected (b). One-half of the disk was mounted for sectioning perpendicular to the articular surface (c). From the other half, a 2-mm-diam plug was taken and mounted for sectioning parallel to the articular surface (a). Cartilage specimens were cut in a plane parallel to the surface of the cork upon which the samples were mounted. The resulting fibril orientation for sections cut parallel to the articular surface (tangential zone) are shown in panel (d). Fibrils are in a two-dimensional plane parallel to the articular surface. The fibril orientation for the sections cut perpendicular (e) includes the tangential, transitional, and radial zones described in Fig. 1. Panels (f) and (g) schematically show the relationship of the ultrasonic wave direction to the perferred fibril orientation in parallel and perpendicular sections, respectively.

B. Scanning laser acoustic microscope

A scanning laser acoustic microscope (Sonomicroscope 100®, Sonoscan, Inc., Bensenville, IL 60106), operating at a frequency of 100 MHz, is used to determine the attenuation coefficient and speed of ultrasonic propagation through adult bovine articular cartilage. The operating techniques of this instrument have been reported in detail.[9,10]

The specimen is placed on a sonically activated fused silica stage along with a thin layer of normal saline and covered with a semireflective coverslip. A focused scanning laser beam probe detects a dynamic ripple (surface displacement) by reflection from the coverslip surface whose amplitude is proportional to the transmitted acoustic pressure. The laser light transmitted through the specimen and coverslip is processed by the SLAM, producing a two-dimensional image of the specimen on a standard television monitor representing an area approximately 3 mm horizontally by 2 mm vertically.

The attenuation coefficient is determined by an insertion loss procedure,[9] which compares the received signal amplitude of a specimen of known thickness in the sound path with that of the reference medium, normal saline. A subimage area of approximately $400\times250\,\mu$m near the center of the acoustic image (the brightest, most uniform area) is used. To ensure that the acoustic subimage remains at the same location as the detectors, the specimen is placed on a glass slide that is coupled to the acoustic wave from the SLAM stage with saline. The signals received from the subimage area are digitized to yield an average amplitude value (V). A minimum of five V values is recorded for normal saline, the reference medium. The glass slide is then moved and a minimum of three values of V is recorded at each of five separate locations within the specimen subimage area. An insertion loss (IL) value, in dB, was calculated using

$$\mathrm{IL} = V_s - V_r, \tag{1}$$

where V_s is the average of V recorded for normal saline and V_r is that for the cartilage. The slope of the linear least-squares fit line for an IL versus thickness plot yields the attenuation coefficient.

Speed[10] is determined from the interference-mode image of the SLAM. The field of view (3×2 mm) contains approximately 39 vertical interference lines equally spaced about $85\,\mu$m apart and covers the specimen and the reference medium of known ultrasonic speed. The speed of sound was determined by the horizontal shift in the fringe lines between the reference medium and the specimen.

Smaller regional differences can be distinguished with the speed analysis than with attenuation coefficient analysis. Each speed pixel is approximately $4\times8\,\mu$m, and a speed profile (80×2 mm) along the vertical direction of the section is generated. In contrast, each insertion loss pixel is $400\times250\,\mu$m, and an average IL value [Eq. (1)] per section is generated over a region approximately 1 mm in extent. Further, at least three separate sections are required to calculate the attenuation coefficient.

Uncertainty in the SLAM assessment of wave speed and attenuation coefficient was determined for solutions of known acoustic properties and duplicate samples of skin and healing wound tissue. The details of these measurements have been reported elsewhere.[11] When a homogeneous medium was used, the accuracy (proximity to the true value) was $\pm$ 2.9%, and the precision (reproducibility of successive independent measurements) was $\pm$ 0.4% for speed, and for attenuation coefficient, the accuracy and precision were $\pm$ 12% and $\pm$ 15%, respectively. These accuracy values represent the accuracy of the SLAM. When heterogeneous samples of normal skin and wound tissue were used, the speed and attenuation coefficient precisions were $\pm$ 1.7% and $\pm$ 16%, respectively. Since the degree of heterogeneity of the articular cartilage samples was not as great as that of normal skin and wound tissue, a worst-case estimate of precision for the measurements reported herein would be $\pm$ 1.7% for speed and $\pm$ 16% for attenuation coefficient.

Statgraphics®, a statistical graphics system, was used to determine the significance of both attenuation coefficient and ultrasonic speed versus treatment and fibril orientation. A multifactor analysis of variance (ANOVA) was performed to assess the effects of tissue properties (treatment, section, and fibril orientation) on ultrasonic properties (attenuation coefficient and ultrasonic speed) and the least significant difference (LSD) range test was used to assess homogeneous groups.

C. Orientation nomenclature

The terms "parallel section" and "perpendicular section" refer to whether the specimen was sectioned parallel or perpendicular to the articular surface, respectively, as represented in Fig. 2. In addition, three fibril orientation terms were defined, which describe the relation between the sound beam direction and the fibril orientation: (1) *Across*, where the sound beam direction is perpendicular to the fibril axis. For parallel sections, the sound beam direction is predominantly across the fibril axis in the tangential zone; whereas in the perpendicular sections, the sound beam is across the fibril axis in the radial zone [see Fig. 2(f) and (g)]. (2) *Along/across*, where the fibril axes vary from perpendicular to parallel to the sound beam direction. For the perpendicular section, fibrils in the tangential zone are both perpendicular and parallel to the sound beam direction and at intermediate orientations [see Figs. 1 and 2(g)]. (3) *Random*, where the fibril orientation is random relative to sound beam direction. For the transitional zone of the perpendicular section, the sound wave tends to encounter more randomly oriented collagen fibrils (see Figs. 1–3).

II. RESULTS

Table I lists the attenuation coefficient and standard error for all 16 cartilage samples. The most consistent and striking difference in attenuation coefficient is seen between the collagen only and the cross-links cleaved samples. These samples contain essentially the same amount of collagen, but most of the intermolecular cross links of collagen have been functionally disrupted in the latter, as shown diagrammatically in Fig. 1. Disrupting intermolecular cross links doubled the attenuation coefficient of both parallel and perpendicular sections. Removing glycosaminoglycans, proteo-

TABLE I. Attenuation coefficient (dB/mm) ± s.e. at 100 MHz for perpendicular and parallel sections for duplicate samples.

	Sample	Perpendicular	Parallel
Normal	A	88 ± 9	92 ± 10
	B	105 ± 11	147 ± 15
CS-depleted	A	108 ± 6	40 ± 4
	B	112 ± 6	63 ± 3
Collagen only	A	75 ± 9	77 ± 14
	B	85 ± 5	90 ± 5
Cross-links cleaved	A	143 ± 14	166 ± 14
	B	185 ± 14	165 ± 15

glycans, and other noncollagenous proteins appeared to have a less predictable effect on attenuation. In perpendicular sections, the CS-depleted samples attenuated the ultrasonic energy slightly more than did normal or collagen only samples, but in parallel sections, the CS-depleted samples attenuated much less than normal and collagen only samples. Also, CS-depleted samples attenuated ultrasound much more in perpendicular than in parallel; whereas, for the other preparations, there was little distinction between perpendicular and parallel.

The most complete speed data came from perpendicular samples (see Table II). Because of smaller pixel size, speed data could be obtained for perpendicular sections from three anatomical regions of articular cartilage (tangential, transitional, and radial). The means ± s.d. of the ultrasonic speeds in these three domains of eight samples are listed in Table II. Although actual values varied greatly between individual sections (hence, the large s.d.), the speed profile across a section was consistent. Figure 3 shows a typical speed profile for a perpendicular section. The highest speed value was seen in the radial zone where the fibrils are oriented mainly perpendicular to the articular surface (across). Speed dropped significantly in the transitional zone (random), then tended to rise again slightly in the surface, tangential zone (along/across). The differences in wave speed among anatomic zones, we believe, related to the difference in fibril organization. In the transitional zone, fibrils are essentially randomly oriented in three dimensions. In the tangential zone, fibrils mostly run parallel to the articular surface, but their axes are randomly oriented in the plane of the surface. In particular, going from the transitional zone to the radial zone, the transition from a random to a radial fibril orientation is more gradual than the diagrams in Figs. 1–3 imply.

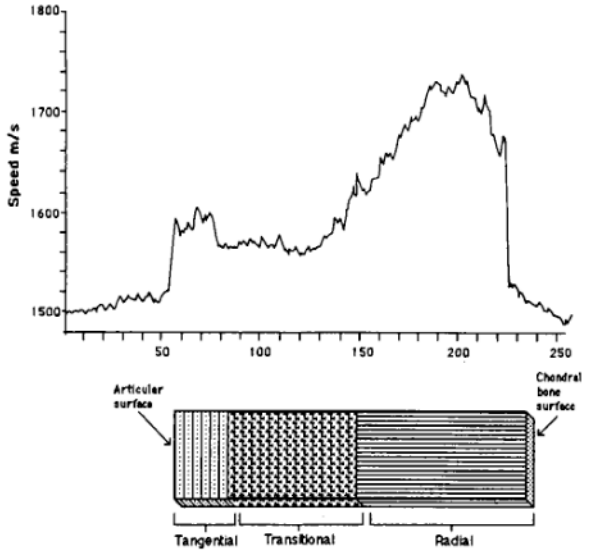

FIG. 3. Profile of wave speed as a function of tissue depth for a slice of normal cartilage cut perpendicular to the articular surface. Changes in the predominant orientiation of fibrils from articular to chondral surfaces are depicted below. The greatest speed is seen in the (radial) zone where sound waves are propagating perpendicular to (across) the long axis of the collagen fibrils (see Fig. 2). The speed is lower at the articular surface (along/across) where propagation is more parallel to the preferred fibril orientation (bearing in mind that the fibrils do tend to be random in the two-dimensional plane of the tangential zone near the articular surface). Speed is lowest in the transitional zone (random). In this transitional zone, the orientation is more random and, as the name suggests, occurs as a gradual transition from one preferred orientation to the other.

Parallel sections were examined only from the tangential zone (Table II and Fig. 2). The direction of sound in these sections was primarily perpendicular to the fibril orientation (across) as shown in Fig. 2(f). The ultrasonic speed in these samples approached that seen in the radial

TABLE II. Ultrasonic speed (m/s) ± s.d. for perpendicular and parallel sections for duplicate samples. Across, along/across, and random refer to the sound beam direction relative to the fibril axis orientation.

		Perpendicular			Parallel
	Sample	Tangential (along/across)	Transitional (random)	Radial (across)	Tangential (across)
Normal	A	1671 ± 61	1647 ± 76	1715 ± 25	1660 ± 44
	B	1635 ± 27	1617 ± 41	1720 ± 99	1690 ± 76
CS-depleted	A	1670[a]	1631 ± 27	1738 ± 51	1775 ± 66
	B	1640[a]	1641 ± 56	1777 ± 114	1793 ± 25
Collagen only	A	1648[a]	1620 ± 38	1704 ± 106	1744 ± 87
	B	· · ·	1653 ± 38	1760 ± 103	1705 ± 74
Cross-links cleaved	A	1650 ± 20	1610 ± 33	1658 ± 53	· · ·
	B	1680[a]	1725 ± 88	1716 ± 9	1695 ± 59

[a]Only one of the thicknesses had a tangential zone from which a speed value could be calculated.

domain of perpendicular sections [also across, Fig. 2(g)]. Hence, ultrasound appears to be propagated more rapidly across the long axis of collagen fibrils than along them.

The ultrasonic speed and attenuation coefficient (dependent variables) were statistically analyzed to estimate the variance in the data explained by each of the following factors (independent variables): Section type (parallel versus perpendicular), anatomic zone (tangential, transitional, and radial), tissue treatment (normal, CS-depleted, collagen only and cross-links cleaved), and orientation (across, along/across, and random). For attenuation coefficient, the anatomic zones and orientation were not evaluated because the pixel subimage ($250\times400\,\mu$m) was larger than each of the separate zones. Hence, the attenuation coefficient results for perpendicular sections represent a spatial average over all three zones and only the factors of section type and tissue treatment were statistically evaluated. The significance of the effect of a factor or the variance of the dependent variable was determined using the F test, where F is calculated as the ratio of the mean-squared variance between each factor mean and the variance within all of the categories of that factor.

The attenuation coefficient was shown not to be significantly different with respect to section type. However, the attenuation coefficient was significantly different with respect to tissue treatment ($F=16.832$, $p\leqslant0.0008$). A multiple-range analysis using the least significant difference range test showed that there were two distinct treatment groupings based on the attenuation coefficient ordering: normal, CS-depleted, and collagen only in one group, and cross-links cleaved in the other group. The attenuation coefficients within the two groups were not significantly different, but those within the first group were significantly different from those within the second. Thus breaking collagen intermolecular cross links resulted in a statistically significant increase in attenuation coefficient, while removing proteoglycans and GAGs plus other noncollagen proteins did not change acoustic attenuation.

ANOVA analyses were also performed on the ultrasonic speed data to determine the effects of section type, anatomic zone, tissue treatment, and orientation. Treatment did not have a significant effect on the ultrasonic speed variation, whereas orientation was found to be significantly different ($F=21.551$, $p<0.0001$). The high significance level and F-ratio support the observation that there is a profound effect of the "preferred" collagen fibril orientation on acoustic speed. Multiple-range tests showed that the across orientation was statistically different from the random and along/across orientations.

III. DISCUSSION

The biochemical effects on cartilage composition of the enzyme treatments used here are well established.[5,6] In CS-depleted samples, 85% of the chondroitin sulfate is removed. Collagen only samples are essentially devoid of noncollagenous matrix, but retain the shape and tensile strength of the original normal sample.[5] From cross-links cleaved samples, about two-thirds of the collagen can be extracted as molecular monomers by nonproteolytic solvents,[6] indicating that most of the intermolecular cross links have been disrupted even though, under physiological conditions, the tissue maintains its original size, shape, and collagen content. While the tensile strength of CS-depleted and collagen only samples is not dramatically altered,[5,12] the cross-links cleaved samples have a 100-fold reduction in tensile strength.[6]

The attenuation coefficient increased markedly in cartilage in which the intermolecular cross links of collagen were disrupted (covalent links broken), compared with control tissue containing a coherent polymeric collagen framework. This observation is the most compelling evidence to date that ultrasonic propagation may be sensitive not only to the amount of collagen present in a tissue, but also to its configuration and degree of cross linking. In addition to this indication of the significance of bonds between type II collagen molecules within individual fibrils, the recent finding that type IX collagen[2] may provide covalent links between fibrils in cartilage matrix[13] could also be relevant, since the elastase treatment will disrupt such bonding.

Acoustic wave speed was most influenced by the orientation of collagen fibrils in specimens. Thus sound speed differed between tangential and radial zones of perpendicular sections in which the predominant fibril orientations were orthogonal to each other (along/across and across, respectively). This observation is supported by the similarity in wave speed between the tangential zone of parallel sections and the radial zones of perpendicular sections, in both of which the sound wave was propagated perpendicular to (across) the dominant plane of fibril orientation[3,4] [Fig. 2(f) and (g)].

It is interesting to observe that a greater percentage of fibrils is oriented perpendicular to the direction of the sound beam for the across orientation than that for the along/across orientation and, in turn, a greater percentage for the along/across orientation than for the random orientation. This is supported by the results in Fig. 3, showing a higher sound speed in the radial zone where the fibrils are predominantly oriented with their axes perpendicular to the sound beam.

Removal of chondroitin sulfate and proteoglycans from the cartilage (CS-depleted samples) apparently had some effect on the acoustic properties, but no clear trend was evident in the few samples studied. Sulfated glycosaminoglycans and proteoglycans bind water and inflate collagenous matrices through the osmotic swelling pressure of their high fixed-charge density.[14] Hence, removing these macromolecules may affect acoustic propagation by perturbing the equilibrium between protein-bound and free water.[15] The tension put on the collagen framework by this swelling pressure may also influence acoustic propagation.

A Brillouin scattering study of the hydration of Li-DNA and Na-DNA films yielded the result that the longitudinal acoustic phonon velocity for propagation perpendicular (across orientation) to the helix axis (called radial velocity) was greater by up to 20% than that of phonons propagating parallel (along orientation) to the helix axis (called axial velocity) for all but the most hydrated speci-

mens.[16] For the degree of hydration (quantified as the film's relative humidity) between 23% and 95%, the radial velocity was consistently greater than the axial velocity. Only for the most hydrated films (98%) was the axial velocity 1%–2% greater than the radial velocity. For the fully hydrated, never-dried articular cartilage specimens (assume 100% relative humidity), the observations are consistent with all but the most hydrated thin-film specimen wherein the speed of the ultrasonic wave propagating perpendicular to the fibril orientation was greater (by about 6%) than that of the wave propagating parallel to the fibril orientation (see Fig. 2).

It may be difficult to compare directly velocity measurements in the gigahertz frequency range where the wavelength ($\simeq 300$ nm) is on the order of the length of the collagen molecule with those at 100 MHz when the wavelength ($\simeq 16\,\mu$m) is much longer, because free water should influence the propagation of sound differently at the two frequencies. In the gigahertz frequency range, the molecular structure more directly affects propagation, whereas at 100 MHz, the characteristic propagation medium probably resembles a composite of collagen and water.

In two separate studies that used the scanning laser acoustic microscope at 100 MHz, wet mouse tail tendon yielded a radial velocity (across) of 1730 m/s (see Ref. 17), whereas wet rat tail tendon yielded an axial velocity (along) of 2200 m/s (see Ref. 18), suggesting that these results are contrary to those of the articular cartilage results reported herein.

Clearly, the mechanisms responsible for acoustic wave propagation in highly organized biological materials are still not well understood. However, it is also clear that careful consideration must be given to orientation as well as to the fundamental intermolecular structure and composition.

Although the number of observations in this series of experiments is small, they give valuable clues on the interaction of ultrasonic energy with soft connective tissues at the ultrastructural and molecular levels. Further studies using a larger number of samples and more complete acoustic and morphological correlations are now needed to confirm and extend these conclusions.

ACKNOWLEDGMENTS

This work was supported by NIH Grants AM 21557, CA 36029, and AR 36794, and Rehabilitation Research and Development Services of the Veterans Administration. We wish to acknowledge the assistance of Marcia Usui with preparation of the figures.

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