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COLLAGEN AS A DETERMINANT OF ULTRASONIC ATTENUATION IN MYOCARDIAL INFARCTS

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I. INTRODUCTION

In previous reports from our laboratory, significantly increased ultrasonic attenuation was demonstrated in regions of scarred myocardium studied 4 to 11 weeks after infarction was produced by coronary occlusion.[1] Although mechanisms responsible for the ultrasonic attenuation of soft tissue have not been adequately defined, the observations by Fields and Dunn[2] and by O'Brien[3] suggest that collagen may be a primary determinant. Because it is well established that increased collagen occurs following coronary occlusion and the evolution of a myocardial infarct,[4] it would appear that increased collagen content might result in increased ultrasonic attenuation in myocardial infarcts. Accordingly, the present study was undertaken to evaluate the relationship between the attenuation of infarcted myocardium and collagen concentration quantitatively.

In this study myocardial infarction was produced in 12 dogs which were killed at either 2 or 6 weeks following coronary occlusion. Investigations were carried out on 38 regions of the hearts from 6 dogs studied 2 weeks after occlusion and on 35 regions from 6 dogs studied 6 weeks after occlusion. Ultrasonic analysis was performed on freshly excised myocardium within 5 to 45 minutes after the death of the animal by transmitting a broadband pulse (2-11 MHz) through the tissue. After ultrasonic analysis was completed, collagen content for each region of myocardium assessed ultrasonically was determined using a quantitative biochemical assay for hydroxyproline concentration. Ultrasonic indices based on the attenuation coefficient versus frequency curve were compared quantitatively to the collagen content of each region.

II. METHODS

A. Animal Preparation

Myocardial infarction was produced in adult, mongrel dogs weighing 15 to 30 kgs. Each dog was intubated after anesthesia with sodium pentobarbital (25 mg/kg, intravenous), placed on a Harvard respirator, ventilated with room air, and subjected to a left thoracotomy via the fifth interspace. The pericardium was incised, and the left anterior descending coronary artery was dissected free immediately distal to the first ventricular branch and ligated. The pericardium was left open, and the chest closed conventionally, with intrapleural suction maintained for at least one hour via a chest tube. Two to six weeks after the operation, each animal was again anesthetized with sodium pentobarbital (50 mg/kg, i.v.). The heart was excised and placed in a 0.9% NaCl solution. To prepare the tissue for ultrasonic analysis, an incision was made at the root of the aorta, continued inferiorly along the left ventricular surface of the interventricular septum to the apex, and extended posteriorly and superiorly, terminating at the base of the heart. This permitted prompt excision of a segment of the anterior and apical wall of the left ventricle. This segment comprised the zone of infarction and surrounding region of normal myocardium in all animals subjected to coronary ligation.

The excised segment of myocardium was mounted on a plexiglass sample holder which maintained the tissue in a stable configuration and did not interfere with ultrasonic analysis. The mounted sample was then placed into a 0.9% saline bath maintained at 20°C, a temperature selected to minimize the effects of tissue degradation. This temperature was shown to provide reproducible results for measurements made up to several hours after excision.[5] Ultrasonic analysis was conducted on discrete regions of tissue of 2 mm in diameter. Previous control studies indicated that biopsies of 1.0 cm diameter were required to provide sufficient tissue to yield reliable and reproducible values of collagen content from the biochemical assay. Accordingly, three ultrasonic measurements were conducted within each 1.0 cm diameter tissue region. Results of the three ultrasonic measurements were averaged to represent the ultrasonic properties corresponding to each 1.0 cm biopsy. After completion of the ultrasonic studies, the sample holder and tissue were removed from the bath. A plexiglass plate with openings spaced to correspond to the discrete 1.0 cm diameter sites was placed over the tissue to assure that each biopsy obtained for collagen analysis corresponded precisely to the region analyzed ultrasonically. The wet weight of each biopsy was determined, and the tissue was frozen immediately in liquid nitrogen with storage at -20°C. Ultrasonic analysis was initiated within 5 minutes following the death of the animal, and the entire process was

completed within 45 minutes.

B. Ultrasonic Methods and Data Analysis

The instrumentation employed for ultrasonic analysis has been described in detail elsewhere.[1] Broadband (2-11 MHz) pulses were transmitted through the tissue and detected by a pair of identical focused piezoelectric transducers (1.3 cm diameter, 5 cm focal length, 5 MHz nominal center frequency). Focused transducers were utilized to minimize possible errors associated with phase cancellation effects. The ultrasonic pulse detected at the receiving transducer was fourier analyzed using an analogue spectrum analyzer and processed digitally. The attenuation was quantitated using a substitution technique by which the signal loss resulting from the transmission through a known thickness of tissue was compared to the signal loss resulting from the transmission through the same thickness of saline.[6] Although data were recorded and analyzed over the frequency range 2 to 11 MHz, certain parameters derived from the attenuation coefficient versus frequency curve were calculated for the range 4 to 9 MHz because the ratio of the signal loss to noise was largest and most consistent over this more restricted range.

Previous studies from our laboratory have demonstrated that artifacts appear in the apparent attenuation coefficient because of ultrasonic phase cancellation effects.[1,7] Although errors arising from phase cancellation effects can be minimized by placing the specimen entirely within the focal zone of focused transmitting and receiving transducers, they can not be eliminated completely. Therefore, the extent to which phase cancellation effects degraded attenuation-frequency data in the present study was estimated with the statistical index root mean square deviation previously described.[1] The root mean square deviation (RMSD) is defined with respect to a least squares line fit to the attenuation coefficient versus frequency curve. On the basis of data obtained using a phase cancellation insensitive acoustoelectric receiver, attenuation coefficient-frequency curves in the present study exhibiting RMSD's greater than 0.25 dB were deleted from further consideration. The value 0.25 dB represents an RMSD which is two times the maximum RMSD exhibited by data obtained on normal myocardium using the acoustoelectric receiver.[1,7] The result of applying this data acceptance criterion in the present study was to eliminate from further consideration 6% of the ultrasonic data from myocardium studied 2 weeks after occlusion and 7% of the data from myocardium studied 6 weeks after occlusion.

In order to compare the results of the ultrasonic measurements with the results of the biochemical determination of collagen content, it was convenient to characterize the ultrasonic attenuation coefficient data over a range of frequencies by a simple index.

Accordingly, two ultrasonic indices were computed. One index used was the slope of a least squares line fit to the attenuation coefficient versus frequency data over the range 4-9 MHz. A second index, designated the slope difference (ΔS), was defined as the difference between the slope of the attenuation of a particular site and the average slope of the attenuation for all normal sites from the same heart. Thus a value of ΔS greater than zero corresponded to a region of increased attenuation over that of normal regions in a particular heart and a value of ΔS less than zero corresponded to a region of decreased attenuation.

C. Determination of Collagen Content

Within one week after ultrasonic analysis, the frozen samples were homogenized in distilled water, and the residue taken to constant dry weight at 100°C. After acid hydrolysis and filtration of the sample, hydroxyproline concentration of the supernatant was determined as described by Kivirikko, Laitinen, and Prockop.[8] After neutralization of the hydrolysate, oxidation of hydroxyproline to pyrrole was accomplished by chloramine-T. Oxidation was terminated by sodium thiosulfate and the pyrrole was extracted with toluene. Following addition of Erlich's solution, quantitative hydroxyproline was calculated from the optical density observed at 560 nm by comparison with results observed for appropriate standard solutions. Based on previous studies, collagen content may then be calculated from the hydroxyproline concentration.[9] In the present study, data obtained from the hydroxyproline assay are presented as percentage of collagen per unit wet weight.

Although the principal source of hydroxyproline in myocardial tissue is known to be collagen, it has been reported that a small amount of hydroxyproline may be derived from elastin, the only protein other than collagen containing hydroxyproline. In order to determine what fraction of hydroxyproline in myocardium was derived from collagen and what fraction from elastin, additional control studies were undertaken. Elastin content was determined by an elastin-specific assay for desmosine,[10] and collagen content was determined by amino acid analysis.[11] Results of these independent assays revealed that elastin gave rise to less than 3.0% of the total hydroxyproline concentration in either regions of normal myocardium or in regions of infarct. Thus, total hydroxyproline concentration appears to be an acceptable measure of collagen content.

III. RESULTS

In order to document the range of collagen concentration and the ultrasonic properties of normal myocardium, 11 regions of myocardium from 3 dogs not subjected to coronary occlusion were analyzed ultrasonically and biochemically. The average ultrasonic

attenuation coefficient at frequencies over the range 2 to 11 MHz and the average slope of the attenuation for these 11 regions were consistent with values for the average attenuation and slope of the attenuation of normal myocardium previously reported from this laboratory.[5,12] Results of the hydroxyproline assay for the 11 regions indicated that the average collagen concentration for normal myocardium was 0.75% of wet weight, with a standard deviation of 0.15%. Based on these results, regions of myocardium exhibiting collagen concentrations of less than 1% of wet weight (approximately the mean +2 S.D.) were designated normal (i.e., non-infarct).

To investigate changes in the ultrasonic properties of myocardium occuring after infarction, 38 regions from 6 dogs were studied 2 weeks after occlusion and 35 regions from 6 other dogs were studied 6 weeks after occlusion. Figure 1 illustrates the range of collagen concentration exhibited by regions from all hearts studied. The percentage of sites exhibiting a specified concentration of collagen is plotted as a function of collagen concentration for 3 groups: i) sites of infarct from hearts studied at 6 weeks (upper panel), ii) sites of infarct from hearts studied at 2 weeks (middle panel), iii) normal sites obtained from non-infarct regions of hearts studied at both 6 weeks and 2 weeks. The concentration of collagen in regions of infarct from hearts studied at 6 weeks was generally higher than that in regions of infarct from hearts studied at 2 weeks. The average collagen concentration of regions of infarct from hearts studied at 6 weeks was 4.9% ± 0.5% of wet weight (mean ± S.E.) compared with an average collagen concentration of 2.9% ± 0.2% for regions of infarct studied at 2 weeks.

As illustrated in Figure 1, there were no sites in the 2 week group which exhibited a collagen concentration greater than 5.5% of wet weight. Based on this observation, all myocardial regions studied were segregated into three groups: i) collagen concentration less than 1%, defining the range of normal myocardium, ii) collagen concentration greater than 1% but less than 5.5%, representing sites of infarct of intermediate collagen concentration, and iii) collagen concentration greater than 5.5%, representing sites of infarct with markedly elevated concentration. The average attenuation coefficient at 2, 4, 6, 8, and 10 MHz and the slope of the attenuation computed over the range 4 to 9 MHz in each of the three ranges of collagen concentration defined above are presented in Table I for hearts studied at 6 weeks and 2 weeks. The results presented in Table I indicate that the average attenuation of normal sites (collagen concentration less than 1%) is nearly identical for dogs studied at 2 and 6 weeks. Similarly, the average attenuation exhibited by sites with collagen concentration in the range 1 to 5.5% is essentially the same for hearts studied at 6 weeks and 2 weeks. These observations suggest that the ultrasonic attenuation data are directly related to the collagen concentration independent of the time interval following the ischemic

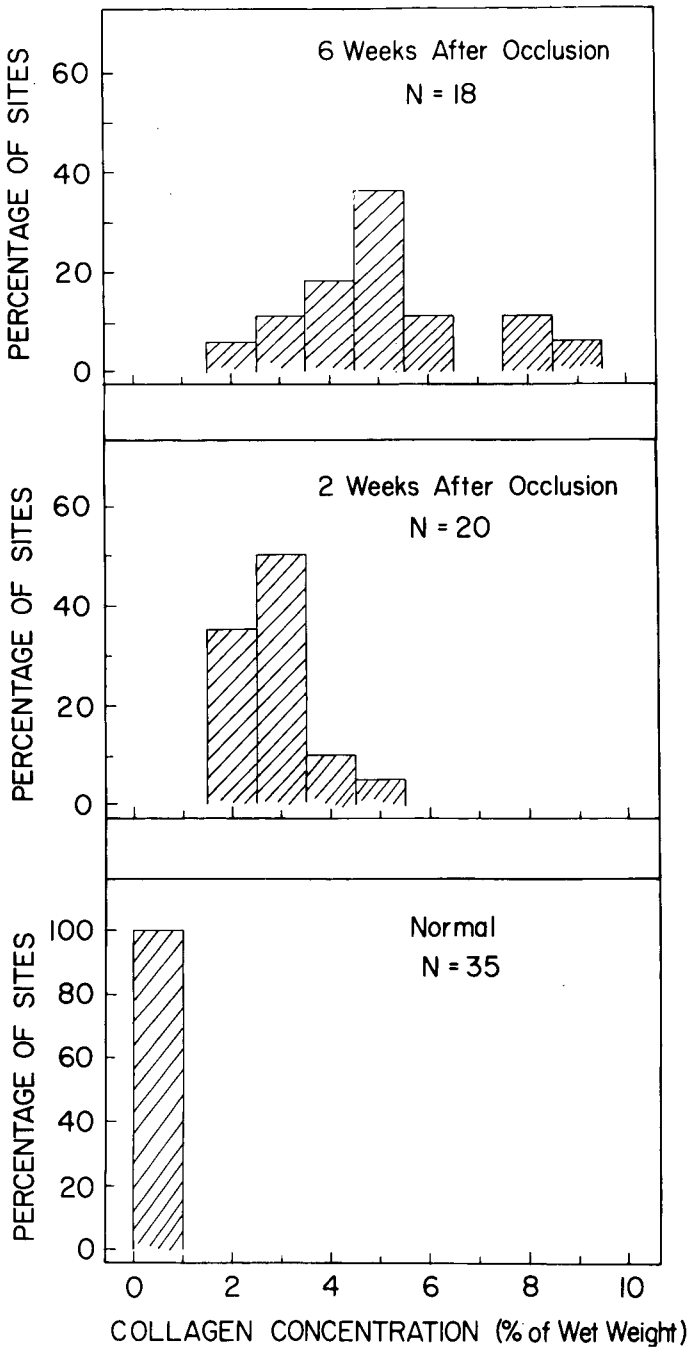

Figure 1. The range of collagen concentration is illustrated for 3 groups: sites of infarct studied at 6 weeks, at 2 weeks, and non-infarct sites at both 6 and 2 weeks.

Table I. Average ultrasonic attenuation in dog myocardium at several concentrations of collagen

Description	Number of Sites	Number of Dogs	Attenuation Coefficient (cm^{-1}) (mean ± S.E.) 2 MHz	4 MHz	6 MHz	8 MHz	10 MHz	Slope of α-vs-freq. (cm^{-1}-MHz^{-1})
Six Weeks After Occlusion:								
Collagen <1% (Normal)	18	6	0.10 ± 0.02	0.20 ± 0.02	0.33 ± 0.03	0.48 ± 0.03	0.64 ± 0.04	0.075 ± 0.002
1% < Collagen < 5.5%	12	6	0.16 ± 0.02	0.25 ± 0.03	0.42 ± 0.03	0.63 ± 0.04	0.78 ± 0.05	0.095 ± 0.006
Collagen > 5.5%	5	6	0.19 ± 0.03	0.34 ± 0.04	0.59 ± 0.06	0.90 ± 0.07	1.20 ± 0.11	0.158 ± 0.010
Two Weeks After Occlusion:								
Collagen <1% (Normal)	18	6	0.10 ± 0.02	0.20 ± 0.02	0.32 ± 0.03	0.47 ± 0.03	0.63 ± 0.04	0.073 ± 0.002
1% < Collagen < 5.5%	20	6	0.14 ± 0.02	0.24 ± 0.03	0.40 ± 0.03	0.59 ± 0.04	0.75 ± 0.05	0.095 ± 0.003

insult. Accordingly, the data were grouped by collagen concentration, combining the results from 6 week and 2 week hearts. The average attenuation properties exhibited by the data combined in this way are presented in Figure 2. The average slope of the attenuation over the range 4 to 9 MHz was (0.158 ± 0.010) cm^{-1} MHz^{-1} (mean ± S.E.) for sites of infarct with collagen concentration greater than 5.5%. The average attenuation for sites of infarct with collagen concentration in the range 1 to 5.5% was (0.095 ± 0.003) cm^{-1} MHz^{-1} and (0.074 ± 0.002) cm^{-1} MHz^{-1} for normal sites exhibiting collagen concentration less than 1%.

The data presented in Figure 2 suggest that the ultrasonic attenuation increases with increasing collagen concentration. To examine this hypothesis further, the relationship between collagen concentration and increased attenuation relative to that of normal was investigated. The specific ultrasonic index examined was the slope difference (ΔS), defined as the change in the slope of the attenuation at a particular region measured relative to the average slope of the attenuation for all normal regions of myocardium from that animal. In Figure 3 the slope difference is displayed as a function of collagen concentration for all regions of myocardium studied at 6 weeks. As can be seen the slope difference increases with increasing collagen concentration, with a correlation coefficient of 0.90. A similar relationship was found in dogs killed two weeks after occlusion, but with a lower correlation coefficient of 0.74. The linear regression fit between slope difference and collagen concentration for data from hearts studied at 2 weeks was nearly identical to that for data from the hearts studied at 6 weeks.

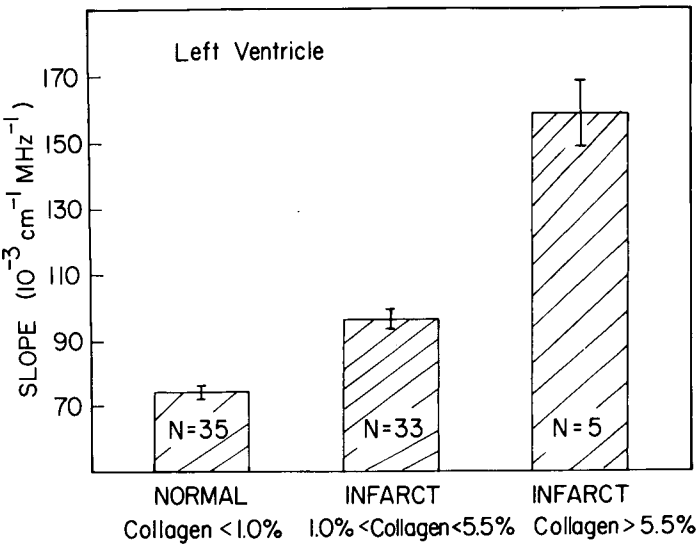

Figure 2. The average slope of the attenuation is illustrated for three groups of myocardial regions, where the groups are classified according to collagen content.

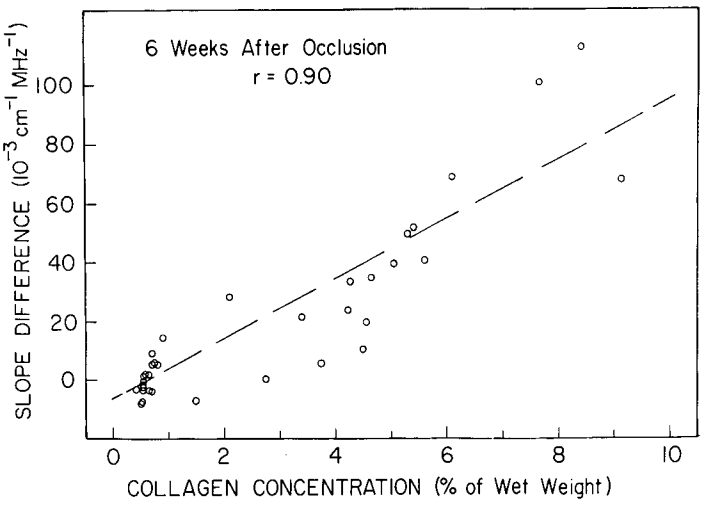

Figure 3. The ultrasonic index slope difference is plotted as a function of collagen concentration for myocardial regions studied 6 weeks after coronary occlusion.

IV. DISCUSSION

This study was designed to examine the relationship between the ultrasonic attenuation and the concentration of collagen in myocardial tissue. Control studies conducted on myocardium from dogs not subjected to coronary occlusion established that the collagen concentration of normal tissue was 0.75% of wet weight, with a standard deviation of 0.15%. In contrast, regions of myocardial infarct from hearts studied 2 weeks and 6 weeks following coronary occlusion exhibited collagen concentrations ranging as high as 9% with an average value of 4.9% in hearts studied at 6 weeks, and ranging as high as 5.5% with an average value of 2.9% in hearts studied at 2 weeks. Based on these observations, ultrasonic data from dogs subjected to coronary occlusion were grouped according to collagen concentration. The results presented in Table I and Figure 2 indicate that sites with collagen concentration less than 1% exhibit the lowest ultrasonic attenuation, sites with collagen concentration between 1% and 5.5% exhibit somewhat higher attenuation, and sites with concentration greater than 5.5% exhibit substantially increased attenuation. Thus, in a qualitative way, the ultrasonic attenuation is observed to increase with collagen concentration.

To evaluate this apparent relationship in a more quantitative way, the results of ultrasonic and biochemical measurements at individual sites were compared. The results presented in Figure 3 indicate that increased attenuation correlated well ($r = 0.90$) with increased collagen concentration for regions of myocardium studied 6 weeks after coronary occlusion. A similar relationship between increased attenuation and collagen concentration was found in myocardium studied 2 weeks after coronary occlusion, but with a lower correlation coefficient of 0.74. The lower correlation coefficient associated with animals studied at two weeks appears to be related to experimental errors inherent to these measurements. The magnitude of these errors can be estimated by noting the variance in the slope difference for all normal sites (i.e., sites with collagen concentration less than 1% of wet weight) in Figure 3. In dogs studied 2 weeks after occlusion the average collagen concentration of regions of infarct is significantly lower than the average concentration in dogs studied at 6 weeks. In addition, the average attenuation of regions of infarct is lower in myocardium studied at 2 weeks as compared to myocardium studied at 6 weeks. Consequently, experimental errors are fractionally more important in the analysis of myocardium from dogs studied 2 weeks after occlusion.

Additional evidence for a relationship between increased attenuation and collagen in remote myocardial infarcts is provided by the exact form of the functional relation between the variables, slope difference and collagen concentration. As illustrated in

Figure 3, the slope difference is an increasing, and approximately linear, function of the collagen concentration. In addition, the linear regression fits between these variables are nearly identical for myocardium studied at 2 weeks and at 6 weeks following coronary occlusion. This result strongly suggests that the mechanisms responsible for the attenuation in regions of infarct are similar at two weeks and at six weeks following the ischemic insult. Thus, the results of this study indicate that the increased attenuation observed in regions of myocardial infarct two to six weeks after coronary occlusion in the dog is directly related to collagen deposition in the tissue.

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