Assessment of fibrosis in infarcted human hearts by analysis of ultrasonic backscatter

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ABSTRACT In animal hearts, the magnitude of integrated ultrasonic backscatter is increased in fibrotic myocardium. Our purpose in this study was to quantitate the relationship between ultrasonic backscatter and collagen deposition in 10 excised human hearts with old infarcts. A 2.25 MHz, 50% fractional bandwidth transducer was positioned at the transducer focal distance from the epicardium of each specimen. The radio frequency backscatter signal was digitized, squared, and integrated to yield the integrated ultrasonic backscatter, which was referenced to the backscatter from a water/steel interface. The interrogated myocardium was then excised and divided into two portions. One portion was assayed for hydroxyproline, a marker for collagen. A second portion was sectioned, stained with Masson’s trichrome, and studied with the use of a computer-assisted image analysis system. There was a linear correlation between the magnitude of integrated backscatter and myocardial collagen content estimated by hydroxyproline assay ($r = .78$). Quantitative histologic analysis revealed a variable relationship between the transmural distribution of collagen and the corresponding transmural pattern of the backscatter signal. In two specimens exhibiting a discrete layer of subendocardial fibrosis, the backscatter amplitude was also increased in the subendocardial region. In specimens with other patterns of fibrosis, the local backscatter amplitude did not correspond to the transmural pattern of collagen distribution. We conclude that the quantitative analysis of ultrasonic backscatter shows promise for the noninvasive evaluation of myocardial fibrosis after infarction.


COLLAGEN deposition is a stereotyped response to myocardial infarction and other types of myocardial disease. For antemortem diagnosis of myocardial fibrosis, the electrocardiogram, echocardiogram, radionuclide image, and endomyocardial biopsy have been used. However, accurate quantitation of fibrotic changes may be difficult by these methods. Thus, determination of the presence and extent of myocardial fibrosis remains an unsolved clinical problem.

Several recent observations have suggested that the quantitative characteristics of reflected ultrasound may be used to infer the presence of myocardial fibrosis. Specifically, in dog and rabbit hearts Mims et al. found that integrated ultrasonic backscatter was increased in fibrotic myocardium. Since measurements of ultrasonic backscatter may be obtained through the intact chest, noninvasive detection of myocardial fibrosis in patients might be possible as well. Pursuant to this goal, the purpose of the present study was to examine the quantitative relationship between integrated ultrasonic backscatter and collagen in infarcted human hearts. In a previous study, we reported the 2.25 MHz integrated ultrasonic backscatter in normal human ventricles, which have a narrow range of collagen content and uniform backscatter amplitudes. For the current study, we postulated that in fibrotic myocardium (1) the magnitude of integrated ultrasonic backscatter would be related to the collagen content of the myocardium, and (2) foci of collagen deposition would result in spatially localized increases in backscatter amplitude.

Methods

Summary of protocol. Ultrasonic backscatter measurements were obtained in vitro from 15 left ventricular free wall specimens of 10 adult human hearts. Using a 2.25 MHz center frequency transducer, the radio frequency (RF) backscatter signal from each specimen was quantitated and referenced to the signal obtained from a water/steel interface. The segment of
myocardium corresponding to the ultrasound sample volume was excised and divided into portions for hydroxyproline assay and, in grossly fibrotic regions, for trichrome-stained histologic examination. The relative area of connective tissue (defined here as the normal interstitial area plus any abnormal collagenous tissue) and its transmural distribution were quantitated from the histologic sections with a computer-assisted method of image analysis. The ultrasound amplitude and transmural pattern were then compared with the collagen (hydroxyproline) content and transmural distribution in each specimen.

**Tissue preparation.** Ten human hearts with grossly evident fibrotic changes and associated coronary artery disease, but without evidence of acute infarction, were obtained at autopsy. Routine postmortem examination included determination of heart weight, dissection of the coronary arteries and portions of the ventricles, immersion fixation in 10% acetic-buffered formalin, and preparation of routine hematoxylin and eosin-stained sections. We have previously demonstrated in dog hearts that the 2.25 MHz integrated ultrasonic backscatter from freshly excised left ventricles is not significantly altered by 10% formalin fixation.7

A heart was selected for this study if an intact 5 × 5 cm or larger specimen of the left ventricular free wall containing gross pathologic evidence of fibrosis (wall thinning, discoloration, or stiffness) was found. In five hearts, a second intact 5 × 5 cm or larger specimen of the left ventricular free wall, in the distribution of a different coronary artery, was also available. Grossly, the latter five regions had minimal fibrosis. Thus, a total of 15 left ventricular specimens exhibiting a wide range of fibrotic changes were obtained from the 10 hearts.

Each specimen for ultrasonic study was mounted in a plastic frame. A 2.0 × 0.4 cm region was then identified in the central portion of each specimen by marking a single row of five inkspots separated by 4 mm (the approximate 3 dB ultrasound beam width) on the epicardial surface along the plane of the short axis of the heart (see Ultrasonic measurements below and figure 1).

After ultrasonic measurements were obtained, the 2.0 × 0.4 cm region was excised en bloc and divided in half from epicardium to endocardium through the inkspots (beams centers). The apical portion was further dissected by removing the epicardial layer corresponding to the epicardial specular reflection. Next, the portion of mural myocardium corresponding to the length of an electronic gate (see Ultrasonic measurements below) was excised and stored in 10% formalin for subsequent hydroxyproline assay.

In the 15 grossly fibrotic specimens, the basal portion was embedded in paraffin, sectioned at a thickness of 8 μm, and stained with Masson’s trichrome. This histochemical procedure is specific for collagen fibers, which stain green (muscle fibers stain red).8

**Ultrasonic measurements.** The mounted ventricular specimens were immersed in a water bath at 22°C, with the epicardial surface at the focal length (6.1 cm) of a 1.27 cm diameter, 2.25 MHz center frequency, and 50% fractional bandwidth transducer (Panametrics Corp., Waltham, MA). The specimen was oriented to obtain the maximal epicardial specular reflection, which occurred when the epicardium was parallel to the transducer face. A stepless electronic gate (Panametrics Corp.) was used to display the ultrasonic backscatter for the time period starting at the end of the epicardial specular reflection and continuing until the high-amplitude deflections at the endocardial interface were noted (transmural gating). Thus, gate length varied with wall thickness. Also recorded was the time between the initial deflection of the epicardial specular reflection and the start of the stepless gate. This permitted calculation of the position of the time gate with respect to the epicardium based on a velocity of sound in myocardium of 1.57 mm/μsec, or approximately 0.8 mm of myocardium per microsecond.9

The gated RF ultrasound signal was amplified, displayed on an oscilloscope (at 1 μsec/horizontal division sweep speed), and recorded on Polaroid 667 film. We have previously described in detail our method for analysis of these photographs.7 In the present study, the backscatter RF waveforms were digitized, squared, and integrated to yield the backscattered energy for the total transmural specimen. To correct for the differences in gate length (wall thickness) among specimens, the total backscattered energy was divided by the gate length to yield backscattered energy per microsecond (E_{myocardium}). With a mechanical guide to center the transducer over each of the five inkspots, ultrasonic measurements of energy per microsecond were obtained at each position. For each ventricular region, these five measurements of backscatter were averaged to reduce fluctuations resulting from phase cancellation artifact, which yielded the average energy per microsecond for each region (E_{myocardium}). The effects of attenuation with increasing depth in the ventricular wall were neglected.

For each specimen studied, a reference energy (E_{steel}) was obtained from the surface of a stainless steel plane reflector substituted for the tissue. Integrated backscatter for each region was then calculated in decibels less than the signal from the water/steel interface by the formula

\[
\text{Integrated backscatter} = 10 \log \left( \frac{E_{\text{myocardium}}}{E_{\text{steel}}} \right)
\]

**FIGURE 1.** Drawing of an excised specimen from the left ventricle. Five inkspots were placed in the central portion of the specimen to identify a region of interest for ultrasonic study. The electronic gate length corresponded to the segment of myocardium subsequently excised for histologic study (indicated by bracket). The interrogated myocardium was removed en bloc and divided (along the line through the inkspots) into equal portions for histologic study and hydroxyproline assay. The short parallel lines denote 3 μsec thick layers of myocardium. Tissues corresponding to the epicardial and endocardial specular reflections were discarded. LAD = left anterior descending coronary artery.
To evaluate the transmural pattern of backscatter, the original RF signal was also divided into 3 μsec increments beginning at the epicardium. $E_{\text{myocardium}}$ was derived for each increment, averaged across the five inkspots to yield $E_{\text{myocardium}}$ per increment, and referenced to the steel reflection to give backscatter values in decibels for each 3 μsec interval. When the total RF signal was not evenly divisible by 3 μsec, the backscatter from the remainder was used as a data point only if it was at least 2 μsec in length. These values were compared with local connective tissue concentration, as described below.

**Hydroxyproline assay.** Assay for hydroxyproline content of the 15 myocardial fragments was based on a standard colorimetric technique, which we have also previously used.7, 10 Two previous studies have shown that 10% formalin fixation of myocardium does not influence hydroxyproline concentration.11, 12 Results of the assay were expressed as micrograms of hydroxyproline/milligram dry weight of the tissue. Our results may be compared with others in the literature by noting that approximately one-fourth wet weight equals dry weight and that 7.46 multiplied by hydroxyproline concentration equals collagen concentration.13, 14 Thus

$$\frac{\mu g \text{ collagen}}{mg \text{ wet weight}} = \frac{\mu g \text{ hydroxyproline}}{mg \text{ dry weight}} \times \frac{7.46}{4}$$

**Analysis of histologic sections.** Previous experiments in our laboratory have shown a close correlation between the relative area of connective tissue exhibited in trichrome-stained histologic sections and the collagen content as estimated by hydroxyproline assay.15 For this study, each histologic slide was placed on the stage of a microscope that was interfaced to a video camera (Sierra Scientific Corp., Mountain View, CA). The slide was transilluminated with narrow-band, green-filtered light, resulting in a monochrome (gray scale) image. In the gray-scale image, collagen (and interstitial space) displayed light gray tones, whereas muscle tissue displayed dark gray tones, as previously described.15 The portion of each section that corresponded to the gated ultrasound sample volume was scanned by video camera at low (10 ×) magnification and digitized into a 512 × 512 pixel matrix with 256 gray-level quantization. The relative areas of muscle and collagen were estimated from gray-level histograms that were developed for the overall image and for 2.4 mm (3 μsec) segments from epicardium to endocardium (figure 1). This permitted quantitation of the collagen profile from epicardium to endocardium in a manner analogous to that for the backscatter data in the same tissue.

**Statistical analysis.** Linear regression analysis (least squares method) was used to obtain the coefficients of correlation for (1) integrated backscatter (decibels) vs hydroxyproline content ($\mu g$/mg dry weight), (2) integrated backscatter vs the logarithm of hydroxyproline content (this comparison is appropriate under the assumption that collagen content is related to backscatter coefficient, as opposed to integrated backscatter), (3) the percent area of connective tissue derived from analysis of histologic sections vs the hydroxyproline content, and (4) the percent area of connective tissue in each 2.4 mm layer of myocardium from epicardium to endocardium vs the integrated backscatter for the corresponding 3 μsec period. In addition, the collagen and backscatter profiles from epicardium to endocardium were plotted for qualitative comparison.

**Results**

Collagen content as estimated by hydroxyproline assay varied widely from 5.04 $\mu g$/hydroxyproline/mg dry weight to 52.84 $\mu g$/mg. As expected, histologic study revealed considerable variation in the quantity and location of collagen deposition in the ventricular walls. There was a linear correlation between the magnitude of the integrated ultrasonic backscatter and the hydroxyproline content of the corresponding ventricular region ($r = .78$, $n = 15$, $p < .002$; figure 2). Integrated backscatter was also linearly related to the log of the hydroxyproline content ($r = .81$, $n = 15$, $p < .001$). There was also a close correlation between the percent area of connective tissue derived from computer analysis of histologic sections and hydroxyproline content of the adjacent myocardium ($r = .86$, $n = 10$, $p < .002$; figure 3).

There was a variable relationship between the transmural distribution of collagen (as estimated from the percent area of connective tissue in each 2.4 mm layer of each histologic section) and the corresponding transmural pattern of the ultrasound backscatter signal (that is, the integrated backscatter within each 3 μsec period from epicardium to endocardium). Overall, there was a fair correlation between the percent area of connective tissue and backscatter per 3 μsec increment.
Discussion

The main findings of this study were (1) integrated ultrasonic backscatter was correlated with the amount of collagen in fibrotic human myocardium, and (2) the profile of backscatter amplitude from epicardium to endocardium bore a variable relationship to foci of collagen deposition.

Comparison with previous investigations. Previous studies in animal hearts have shown that ultrasonic backscatter is increased in fibrotic compared with normal myocardium. Our study examined the relationship between backscatter and collagen deposition in human myocardium, and is the first to demonstrate a correlation between these variables. We have previously studied the backscatter and collagen content of normal ventricles, which have a narrow range of collagen content, and found no correlation. This finding suggests that, in normal myocardium, tissues other than collagen are important contributors to ultrasonic backscatter. Similarly, O’Donnell et al. suggested that collagen was not the primary determinant of ultrasonic attenuation of normal myocardium. It is apparent from the data in our current study that, in fibrotic human hearts, collagen content is one of the principal determinants of backscatter.

This study differs from previous work in several important ways. First, a 2.25 MHz center frequency transducer was used since this relatively low frequency is suitable for clinical application. Since backscattered energy increases with frequency, our values for backscatter tend to be substantially less than those reported by others who used higher frequency transducers. Second, we measured the backscatter across the entire transmural thickness of the compact myocardium, exclusive of the specular reflections arising at the epicardium, and at the endocardial trabeculations. This was necessary since wall thickness varies in human hearts and because collagen deposits may be either transmural or differentially located in the subepicardium or subendocardium. Since gate length varied, the backscatter was normalized to wall thickness. Third, only that segment of myocardium corresponding to the gated ultrasound sample volume was excised for hydroxyproline assay. Thus, our experimental design allowed the direct comparison of regional collagen content and regional integrated backscatter.

Relationship between transmural collagen and backscatter profiles. We found that transmural collagen and ultrasound backscatter profiles bore a close resemblance only in specimens exhibiting a discrete layer of subendocardial fibrosis. Specimens with interstitial collagen deposition separated by intact muscle fibers and those with transmural scarring did not yield collagen profiles that were similar to the corresponding backscatter profiles. What may account for this variability in the relationship between focal collagen deposits and spatially localized backscatter amplitudes? The axial (range) resolution of the ultrasound instrumentation we used for this study allowed us to detect subendocardial lay-

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(r = .51, n = 34, p < .005). The comparison plots for transmural collagen and backscatter profiles suggest that the relationship between these variables depends, at least in part, on the pathologic pattern of collagen deposition. In two specimens that exhibited a discrete layer of subendocardial fibrosis, the transmural profiles of collagen and backscatter were similar (figure 4). In the remainder of the specimens, which exhibited either interstitial fibrosis or dense transmural scarring, a poor correspondence was found between local collagen and backscatter, and collagen and backscatter profiles did not appear similar.

FIGURE 4. Transmural collagen and backscatter profiles for a specimen exhibiting dense, subendocardial fibrosis. Top, Values for backscatter (solid line) and collagen (percent area of connective tissue, interrupted line) are shown for each 3 μsec (2.4 mm) layer from epicardium to endocardium. Bottom, Photomicrograph of the trichrome-stained specimen, transilluminated by green-filtered light, from which the profiles were derived. In this case of subendocardial fibrosis, the collagen and backscatter profiles were similar.
ers of collagen. However, the axial resolution was larger than the interstitial collagen deposits, which could not be localized. This fact, along with the presence of phase cancellation artifacts found with phase-sensitive piezoelectric receivers, may explain, in part, our inability to resolve and localize small "islands" of collagen in our study. In the cases of transmural fibrosis, we sometimes found an anomalous decrease in backscatter amplitude toward the subendocardial portion of the profile. This may have been related to acoustic shadowing due to the highly reflective fibrotic tissue. We did not attempt to account for the effects of attenuation in the present study, and we therefore could not correct for this shadowing.

In summary, this study showed that integrated ultrasonic backscatter predicted myocardial collagen content. These data were obtained with an ultrasound transducer suitable for clinical application in patients. The ability of measurements of local, intramyocardial backscatter to predict the spatial pattern of collagen deposition varies with the distribution of fibrosis, appearing most promising in the identification of subendocardial fibrosis. Although much work remains to be done concerning biological and technical contributors to regional ultrasonic backscatter, our data indicate that ultrasonic backscatter may prove to have clinical utility in the determination of the presence and extent of fibrosis after myocardial infarction.

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