In Vivo Quantitative Ultrasonic Evaluation of Myocardial Fibrosis in Humans

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The aim of this study was to assess in vivo whether the regional ultrasonic reflectivity, evaluated by a real-time integrated backscatter analysis, was related to the local content of connective tissue in human myocardium as estimated by quantitative histology of endomyocardial biopsies. Sixteen patients with presumptive diagnosis of cardiomyopathy were ultrasonically studied by means of an M-mode–based echocardiographic system with quantitative integrated backscatter analysis capabilities. A 2.25-MHz transducer was used. The integrated value of the rectified radiofrequency signal of the interventricular septum was taken as integrated backscatter index and expressed in percent normalized for the pericardial interface (assumed to be 100%). All patients also underwent multiple left ventricular endomyocardial biopsies, which were stained with Masson’s trichrome and studied with the use of a computer-assisted image analysis system. The percent integrated backscatter index was significantly higher in the presence of connective tissue area greater than 20% (eight patients) versus less than 20% (eight patients): 51±25% versus 26±11%, p<0.05. A significant correlation (p<0.05, R=0.55) was found between percent integrated backscatter index and percent connective tissue area. In vivo on-line quantitative ultrasound analysis is feasible in man and reliably identifies variations in the regional extent of fibrosis in human myocardium. (Circulation 1990;81:58–64)

Quantitative characterization of myocardial structure by means of analysis of ultrasonic integrated backscatter has been experimentally shown to correlate with the collagen content of the insonified tissue.1–5 An M-mode commercially available echocardiograph was recently developed in our institution for quantitative on-line analysis of backscattered radiofrequency signal coming from myocardial structures.6,7

This approach, based on the time domain analysis of the radiofrequency signals, appears promising as a means to establish certain aspects of ultrasonic diagnosis on a more quantitative basis.3,4 The assessment of regional myocardial fibrosis would be of particular interest since excessive myocardial fibrosis is both an important sign and is associated with a variety of myocardial diseases.

Even though there were substantial problems in comparing exactly the anatomic region interrogated by the ultrasound technique versus the same area sampled by the endomyocardial biopsy, the aim of this study was to assess in vivo whether the regional ultrasonic reflectivity, evaluated by a real-time integrated backscatter analysis, was related to the content of connective tissue in human myocardium estimated by quantitative histology of myocardial biopsy specimens.

Methods

Patient Population

The study group initially consisted of 19 consecutive patients with congestive heart failure and cardiomegaly (seven patients) or suspected cardiomyopathy (12 patients) who underwent left- and right-heart catheterization. The patients ranged from 16 to 65 years old (mean, 44 years). No significant coronary artery or valvular heart disease was found in any patient, and left ventricular biopsy was performed for diagnostic purposes.

Ultrasonic Methods

A block diagram of the data acquisition system is shown in Figure 1. The parasternal window was used in every patient since this approach is the easiest to perform from the technical point of view with the M-mode technique.

An OTE Biomedica 2310 M-mode echocardiograph (provided with an A-mode screen as well) was used for guidance and to provide an electrocardiographic signal. Traditional M-mode echocardiographic technique was used for spatial localization; quantitative analysis of backscattered ultrasound was
performed on the tissue in the region of interest. To collect ultrasonic signals perpendicular to the myocardium, a careful search was made for sites that produced a distinct and persistent specular reflection from the endocardial surface. This practice also aided in accurate placement of the radiofrequency acquisition window. The acquisition of the signal from myocardial walls was always performed at end diastole because it is known that a systematic variation in backscatter amplitude occurs during the cardiac cycle.8

A 2.25-MHz single-frequency transducer (1.3 cm diameter, 7 cm focal distance, 6 cm focal region) was used. The bandwidth of the transducer, measured at −3 dB with respect to the central frequency of 2.25, was 500 KHz. The "native" (raw) radiofrequency signal was sampled before the processing chain of the M-mode instrument (Figure 2). The radiofrequency signal underwent preamplification, bypassing the receiving circuits of the ultrasonic equipment. The analog signal was fed to an amplifier, whose gain sweep (from 2 to 60 dB) was accomplished in 30 steps. Such operation allowed the full use of the input dynamic range of the analog to digital converter. Sampling was performed by a flash converter with 8 bits of amplitude resolution at a rate of 40 MHz. The digitized signal, from analog to digital converter, was stored on fast memories and elaborated by a microprocessor-based system.

The acquisition gate was visualized on the M-mode echocardiographic tracing, the A-mode screen, and the radiofrequency signal to ensure proper positioning. For myocardial analysis the gate length was kept at 3 μsec, which corresponded to 2.35 mm, given the velocity of ultrasound in biologic tissues of 1.57 m/msec. This allowed sampling of the radiofrequency signal well within the myocardium, excluding epicardial and endocardial specular reflections. In particular, for the analysis of the septal and posterior wall, the acquisition gate was kept just behind the specular echo of endocardium (left endocardium for the septum) to minimize the transmural variations in backscatter, depending on the position within the wall from which it was acquired.8 For each ventricular wall the representative value was the average of three values. For pericardial echo evaluation a 3-μsec gate length was also used, centered on the highest echo peak in the time domain. The microprocessor-based analysis involved the measure of integrated amplitude of the rectified signal.

More analytically, the integrated backscatter index (IBI) was calculated as follows: IBI = \int u(t) I dt, where I.I means the absolute value, u(t) = i(t) × s(t), i(t) is the time sequence of backscattered echoes, and s(t) is the time gate delimiting the thickness of the insonated tissue.

During the examination, the IBI value expressed in millivolts (i.e., a linear value) was constantly visualized on a digital display to allow the on-line measurements. Two different methods were used to evaluate septal reflectivity quantitatively: 1) percent IBI, expressed as a comparison with pericardial interface, and 2) compensated IBI, after off-line compensation procedure.

Percent IBI. IBI results for each heart structure were expressed in percent value assuming as 100% the pericardial interface (from which the maximum echo intensity was consistently recorded in end diastole in the individual patient).9,10 The interpatient range in pericardial echo was 40–110 mV. The individual pericardial signal strength was used to normalize myocardial signals in each patient.

Compensated IBI. In this study, a compensation procedure was adopted to overcome problems due to the variable attenuation effects of the interposed...
tissue. The rationale of this approach has been extensively discussed by Melton and Skorton.\textsuperscript{11}

The procedure was performed off line by first evaluating the correction term for each tissue layer that constituted the interposed tissues (each layer was assumed to behave homogeneously as far as frequency-dependent losses) and afterward by multiplying such correction term for the amplitude signal coming from the tissue target (radiofrequency-gated signal). The correction term was evaluated as follows: the thickness of each tissue layer was measured starting from B-mode targeted time-motion tracings; twice each thickness was multiplied by the average attenuation coefficient over the operating frequency range evaluated among the contributions of each interposed tissue layer\textsuperscript{12}; and finally, the exponential amplification was evaluated (i.e., to compensate for the attenuation law), which constituted the correction factor.

Left Ventricular Endomyocardial Biopsy and Histologic Examination

Endomyocardial biopsy was performed during left-heart catheterization using a 6F King bioptome. The tip of the bioptome was positioned under fluoroscopic control on the left ventricular septum close to the inferior wall. Between four and five specimens were obtained from each patient. They measured approximately 3 mm in diameter and were immediately fixed in phosphate-buffered 10% formalin after careful removal from the bioptome.

Previous studies 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.\textsuperscript{13,14} In this study, after completion of routine histologic procedures, the 4–5 μm thick paraffin-embedded sections were stained by Masson’s trichrome stain. Five sections from each specimen were examined. All trichrome-stained sections were placed under a microscope, which was interfaced to a videocamera (Bosch, FRG). Images were digitized at a magnification of 100 on a Mipron (Kontron, Munich, FRG) image analysis system into a 512×512 pixel matrix with 256 gray levels. Each entire section could thus be visualized and its total area automatically calculated. The program for histologic analysis is commercially available in the software of Mipron. For each slide the threshold was identified, which permitted separation of the gray levels corresponding to the red-stained myocardial cells from those corresponding to the blue- or green-stained connective tissue areas (Figures 3 and 4). The percent of overall connective tissue area (interstitial and endocardial) in each section could thus be calculated as the ratio of the number of pixels relative to the blue color to the number of pixels of the entire section. Repeated measurements were performed on each section, and an error below 5% was estimated. The system was validated by comparing its area measurements with those obtained by computer-assisted planimetric determination of the blue- and red-stained areas on sev-
eral images at the same magnification. In this case also an error below 5% was estimated. The average value of percent connective tissue area of the five sections obtained from each specimen was used, and the average value of the four to five specimens for each patient was calculated.

Statistical Analysis

For each variable, the mean value and the standard deviation were reported. Intergroup differences were tested for significance by Student's t test for unpaired values.

Results

Feasibility. From the set of 19 consecutive patients initially considered, one was ruled out because of inadequate echocardiographic imaging with the M-mode instrument, and two further patients were ruled out because of inadequate bioptic sampling. A total of 16 patients had technically satisfactory ultrasound and histologic examinations.

Histologic-ultrasonic correlation. The ultrasonic reflectivity in patients with connective tissue area less than 20% was significantly lower than in patients with connective tissue area greater than 20%: % IBI=26±11 versus 51±25 (p<0.05); compensated IBI=40±15 versus 79±43 (p<0.05). A significant correlation could be found between percent connective tissue area and percent IBI (r=0.55, p<0.05; Figure 5) and between percent connective tissue area and compensated IBI (r=0.53, p<0.05).

Discussion

In vivo quantitative on-line evaluation of ultrasonic backscatter provides a reasonably accurate estimate of histologically assessed fibrosis in human myocardium. These data are consistent with experimental and clinical evidence indicating collagen to be a major determinant of echo intensity.1-5,9,10,15 In particular, Shaw et al15 studied antemortem patients with myocardial fibrosis with a color-encoded echo-

FIGURE 4. Histologic section of bioptic myocardial sample with severe fibrosis. The blue areas correspond to connective tissue.

FIGURE 5. Correlation between percent IBI and percent connective tissue area. One point represents one patient. Each value is averaged over multiple histologic and ultrasonic samplings.
cardiographic system, and they found that the amplitude from septal walls correlated nicely ($r=0.86$) with collagen content of the same regions analyzed biochemically postmortem. However, there are some fundamental differences between Shaw’s study and ours. Their ultrasonic analysis provided a color-coded display of a signal that had undergone conventional processing while with our approach the “native” radiofrequency signal was sampled. With their system, the specular angle-dependent reflectors (coming from endocardium and epicardium) gave a preponderant contribution to the displayed echo, whereas with our approach the backscatter coming from the myocardium was sampled. Finally, the collagen content was estimated with a biochemical method in their correlation with septal amplitude and with a histologic method in ours. It is known that only a weak correlation exists between biochemically and histologically detected fibrosis (see below).

**Limitations of Study**

We agree with Perez et al$^{16}$ that among various methods proposed for ultrasonic tissue characterization, “an approach based on unprocessed radiofrequency signals emanating from tissue provides a particularly robust measurement of the physical state of the tissue.” However, some limitations of the specific methodology employed in the present study should be acknowledged. They are inherent to both ultrasound and histologic evaluation.

The M-mode technique does not allow perfect geometric control of the structure under examination. A narrow frequency bandwidth transducer was used so that a frequency-averaging technique, minimizing phase cancellation artifacts, could not be adopted. However, the ultrasonic sampling was performed on the interventricular septum, a region also easily imaged with the monodimensional technique. From the parasternal approach, this wall also suffers little from variable attenuation phenomena from interposed structures.

Two different approaches were used for quantitative ultrasound examination. In the first approach, the volume backscatter signal was normalized to the magnitude of the specular echo from the pericardial surface to devise an IBI value. The use of this signal forms a self-calibration, eliminating errors due to variable attenuation and diffraction (to first order). Unfortunately, the specular pericardial echo is an extrinsic variable that is sensitive to the details of insonification, such as angle dependence or possible saturation of the front-end electronics from pericardial signals. However, the interobserver and intraobserver reproducibility of this approach has already been demonstrated to be quite satisfactory, and it is relatively simple to perform when compared with the approaches related to phase, frequency dependence, or texture, which have been proposed as possible tools for tissue characterization.$^{3,4}$ The reliability of the recorded differences in ultrasonic septal reflectivity was also substantiated by the fact that similar values of the correlation coefficient with histologically assessed fibrosis were obtained by a totally different ultrasonic quantitative evaluation. With the off-line compensation procedure the pericardial signal was neglected, and therefore, the method did not suffer the limitations inherent in the use of this angle-dependent reference standard. However, this method was more time consuming, required complex off-line data processing, and did not apparently determine any spectacular improvement in the correlation with histologic data. The fact that two separate quantitative ultrasonic methods with different underlying rationales and different limitations gave similar results.

### Table 1. Ultrasonic and Histologic Quantitative Data on 16 Study Patients

<table>
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<tr>
<th>No.</th>
<th>Sex</th>
<th>Age</th>
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<th>Septal % IBIs</th>
<th>Septal compensated IBIs</th>
<th>% Area fibrosis</th>
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seems to confirm the reality of the underlying phenomenon, that is, the increased ultrasonic reflectivity of the fibrotic myocardium.

The histologic examination also suffers some limitations. First, fibrosis is often patchy so that sampling errors are likely to be present. Although samples from histologic and ultrasound estimates came from the same anatomic segment (septoapical region), identical tissue could clearly not be used for both. However, the multiple spatial sampling adopted for both echo and biopsy specimens should have minimized, but surely not cancelled out, this source of error. The myocardial collagen content was estimated by means of a histologic method. Histology depends on the tinctorial properties of the connective tissue elements, which are not well understood. There may be a dissociation between histologic and biochemical (hydroxyproline assay) estimation of collagen content. Mimb et al noted that after experimental myocardial infarction, backscattered amplitude was considerably increased within 6 weeks of experimental infarction, an effect that was abolished by treatment with collagenase although local hydroxyproline values were unaltered. In fact, the hydroxyproline assay is not sensitive to the particular organization state of collagen but merely estimates the total number of collagen molecules present. In contrast, the ultrasonic backscatter is expected to be quite sensitive to the organizational level of collagen. If the fibrils form into bundles, then the resulting structure (i.e., a fiber) is of sufficient size to produce significant ultrasonic scattering. Therefore, the histologic valuation may be even more valuable than biochemical assessment for comparison with ultrasonic backscatter. In a study on infarcted human hearts performed with a 2.25 MHz transducer, Hoyt et al found a fair correlation \( r=0.51 \) between the percent area of connective tissue and backscatter. They concluded that in contrast to what happens in normal myocardium where tissues other than collagen are important contributors to ultrasonic backscatter, collagen content is one of the principal determinants of backscatter in fibrotic human hearts.

Other studies in vitro and in vivo in different pathologic models, such as atherosclerosis, mitral valve disease, myocardial infarction, have shown a good correlation between the amplitude of backscattered echo and histologically assessed collagen. Another important limitation is due to the type of correlation between histologic and ultrasonic data. Fibrosis can be a highly localized and patchy process, both in a transmural as well as a circumferential direction. The transmural variability could be minimized in the present study since both the bioprc and the ultrasound sampling mainly involved the subendocardium. However, the endocardial layer was also encompassed in the bioprc sample, which reached a maximum of 2 mm in depth. On the contrary, the ultrasound sampling accurately avoided the specular

endocardial echo and studied a region of 2–3 mm within the subendocardial layer.

Regarding the myocardial region sampled, the biopsy, which was always performed under fluoroscopic control, explored the inferoseptal region while ultrasound sampled the septoapical region. Therefore, a discrepancy in sampling was unavoidable in at least some cases. The spatial averaging of multiple samples was performed for both histology and ultrasound to limit this bias.

Perspectives

Tissue characterization should ultimately represent a tool for assessing the physical state of the tissue. At least in the model of documented or suspected cardiomyopathy, with the appealingly simple ultrasound technique used, tissue characterization seems on the way to fulfill this promise. It should also be considered that ultrasound information might be implemented in many ways, for instance, by adopting a bidimensional imaging system or by adding a system for rational gain compensation, which might allow one to obtain reliable information from all myocardial structures in conditions of a suboptimal acoustic window.

References


KEY WORDS • echocardiography • tissue • radiofrequency • cardiomyopathies
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