Fibrosis, Lipids, and Calcium in Human Atherosclerotic Plaque

In Vitro Differentiation from Normal Aortic Walls by Ultrasonic Attenuation

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SUMMARY. This study was designed to determine whether attenuation of ultrasound by the aortic wall is potentially useful in characterizing the atherosclerotic lesion. Measurements were made on fresh specimens taken from a human aorta at autopsy. Four hundred different sites, 4 mm in diameter each, corresponding to the dimension of the ultrasonic beam at the focal zone, were ultrasonically analyzed and histologically studied. Attenuation of ultrasound in each site was assessed by Fourier analysis of the echo produced by a specular reflector placed behind the specimen. Two parameters were measured over the range 7–11 MHz: the integrated attenuation index (per cm), and slope (per cm per MHz) of the best fit straight line relating attenuation and frequency. Histological examination—performed for each of the 400 sites where attenuation had been measured—identified four subsets (100 samples each): normal aortic walls, fibrous plaques, fibrofatty plaques, and calcified plaques. Results obtained from ultrasonic and histological analyses showed that the integrated attenuation index was lowest in normal walls (24 ± 2.1, mean ± SE) and progressively increased in fibrous (32 ± 3.1), fibrofatty (82 ± 6.5), and calcific (185 ± 8.7) subsets (all intergroup differences were significant, except for the normal vs. fibrous comparison). The slope value was significantly lower in the fibrous than in the normal subsets: (10⁻³) 31.9 ± 4.5 vs. (10⁻³) 99.5 ± 9.1, respectively. Values of fibrofatty and calcific plaques overlapped: (10⁻³) 383 ± 21 vs. (10⁻³) 320 ± 23, respectively. Both were significantly different from normal and fibrous groups. Thus, alterations in ultrasonic indices related to attenuation characterize the different pathological patterns of atherosclerosis in the human arterial wall, providing a distinction between normal specimens and fibrous, fibrofatty, and calcified plaques. (Circ Res 56: 556–562, 1985)

RECENT information has linked the biochemical profile of atherosclerotic plaque (that is, the relative amounts of fibrous, lipid, and calcific constituents) to its biological activity and clinical relevance. "Unfortunately, however, at our present stage of technology and knowledge, it is virtually impossible to evaluate the quantities of these major components in any given plaque in the human subject at any specific time, short of surgical removal or examination at autopsy" (Wissler, 1984).

In the recent past, ultrasonic tissue characterization has proved useful in the study of many pathological situations (Linzer, 1979; Chivers, 1981). Therefore, we decided to study atherosclerotic disease with this new emerging tool. Encouraging data have been recently obtained in vitro, using backscattered signals as a means of differentiating normal, fibrofatty, and calcific formalin-fixed human aortic walls, both with narrow (Picano et al., 1983a) and broadband (Picano et al., 1983b) transducers. Identification of attenuation properties of normal and diseased arterial tissue could provide additional useful information, since such properties are also based on intrinsic characteristics of the tissue and could be, at least theoretically, measured in vivo exploiting reflected ultrasound (Kuc and Schwartz, 1979; Fink et al., 1983).

The aim of this study was to measure in vitro attenuation properties of human fresh aortic walls—normal, and with different degrees of atherosclerosis. Four hundred aortic regions from human autopic aortas were studied in vitro: 100 normal walls, 100 fibrous, 100 fibrofatty, and 100 calcified plaques. Atherosclerotic disease was independently assessed, after ultrasonic measurements, by histological examination performed on each site. Quantitative indices of ultrasonic attenuation were derived from the frequency dependence of the ultrasonic attenuation characterizing each arterial region over the range 7–11 MHz.
**Methods**

**Experimental Procedure**

Specimens of fresh arterial wall were taken from human aortas at autopsy and were selected to consist of diseased regions containing three distinct kinds of lesion (fibrotic, fibrofatty, and calcified), along with regions—whenever possible—of relatively normal tissue. In order to deal with potential problems of methodology related to the time after death at which the tissues were harvested and examined by ultrasound and the temperature of the tissue during the examination (Mimb's et al., 1979), sampling was performed only when the interval from death to excision ranged between 10 and 15 hours, and the ultrasonic examination was started immediately after excision and performed in a water bath at a constant temperature of 20°C.

Excised samples of aorta were cut down the anterior midline, opened flat, and mounted on a squared, metallic, hollow-rimmed sample holder with pins along its periphery and placed in a distilled water bath. This device provided stabilization of the tissue sample and avoided interference with ultrasonic measurements.

Once the ultrasonic studies were over, the tissue was removed from the water bath, and thickness was measured by means of a micrometer (for each of the sites removed from the water bath, and thickness was measured in a water bath at a constant temperature of 20°C).

For each pathological subset, the first 100 consecutive sites acquired were considered for analysis.

**Ultrasonic Characterization**

Measurements were made by employing the system described in Figure 1. It consists of a single transducer acting as transmitter and receiver of the echo produced by a specular reflector lodged behind the specimen under study. In this approach, the amplitude of the echo that has passed twice through the specimen is measured. The electrical source consisted of a broadband pulser-receiver (Panametric model 5052 PR).

A small-diameter focused transducer (Aerotech, model gamma, 15 MHz nominal frequency, 0.5 cm in diameter, 8 cm focal distance, 4 mm in diameter at the focal zone) was used. The ultrasonic beamwidth (evaluated at -3 dB) was determined with pulsed broadband signal by means of a metallic Rayleigh reflector. To achieve broadband characteristics, we designed this transducer to operate over the frequency range of 4–14 MHz on the left tail of the frequency response of the 15 MHz center frequency quartz plate. Before tissue was placed in the path of the ultrasonic beam, we measured the response characteristics of the system by employing the substitution technique of Schwan and Carstensen (1952). Both the sample holder and the specular reflector were placed in the focal zone of the ultrasonic beam. The mounted tissue sample was analyzed ultrasonically at 16 discrete sites. A square matrix of the 16 sites (4 × 4) was obtained for each specimen, every point being 2 mm apart from the other. For each point, four measurements were obtained with micrometer displacement of the transducer, with the aim to minimize phase cancellation effects (Mimb's et al., 1979). The average measurement of the four was taken as representative of that site. Data collection was performed automatically. Under control of a timing unit, broadband ultrasonic pulses are digitized by a transient recorder (Tektronik, model 7912 AD, conversion rate 10 nsec, 9 bits of amplitude resolution). Data from the transient recorder were continuously transferred to a minicomputer (HP-1000) for the analysis.

**Ultrasonic Indices**

The transfer function (H0) of each specimen was estimated by the ratio of the Fourier transforms of the two signals received, respectively, in the presence and absence of the interposed tissue. The In | H (0 | was evaluated according to the following relationship (Kak and Dines, 1978):

\[
\ln | H_{0} | = \ln T - 2 \alpha_{0} X
\]

where \( \alpha_{0} \) describes the signal loss due both to absorption and scattering of ultrasound within the specimen, \( X \) is the thickness of the sample, and \( T \) represents the product of the amplitude transmission coefficients for the water-tissue and tissue-water interfaces. The attenuation coefficient of the water has been neglected.

The range where \( \ln | H_{0} | \) is a linear function of the frequency (7–11 MHz) has been previously assessed (unpublished data). Over the range 7–11 MHz, two indices were derived:

1. The integral value of \( \ln | H_{0} | \) normalized for the thickness of the specimen (integrated attenuation index: \( A \)). Such an index is supposed to assess both intimal and internal signal losses due to atherosclerosis. The normalization for the thickness is made on both components contributing to \( \ln | H_{0} | \) (see Eq. 1): internal loss (which is thickness dependent) and surface reflection loss (which is thickness independent, and therefore should not be normalized). From the practical point of view, however, it is impossible to discriminate—by analyzing the received signal—one contribution from the other. Therefore, the
normalization procedure introduces an approximation which can be considered reasonable, if it is assumed that a negligible loss in signal occurs due to the reflection at the water-specimen interface (Mimbs et al., 1977).

2. The slope of the least square straight line regression fit to \(1/X \ln |H(f)|\) (slope index: S). Such an index is supposed to be independent of the amplitude transmission coefficients (Kak and Dines, 1978) (In \(A = 0\)).

Equation 1 is based on the assumption that phase cancellation effects are insignificant. This is an obvious oversimplification, which can be considered reasonable, however, when a broadband-focused transducer with a small surface is employed.

Pathological Characterization

Four hundred aortic regions, 4 mm in diameter, equivalent to the dimension of the transducer focal zone, were independently studied, after ultrasonic measurements, by histology. Weigert-Van Gieson stain was employed. According to generally accepted criteria (Robbins and Cotran, 1979), four pathological subsets were identified, each consisting of 100 samples: normal aortic walls, fibrous plaques (i.e., with wall thickened by connective tissue), fibrofatty plaques (usually characterized by a fibrous cap and a lipid core), and calcified plaques (where calcification of the atheroma occurs).

It must be pointed out that each ultrasonically studied aortic region had a diameter of 4 mm, and every point was 2 mm apart from the others. Therefore, a half overlap of the focal zone (corresponding to approximately 30% overlap in two adjacent areas) was made. This is necessary for an accurate acoustic mapping of the specimen. If no overlap had been made, a significant area of the aortic specimen would not have been sampled at all. A greater overlap would have been useful for the acoustic mapping, but this would have made more difficult the correlation with histological data.

Statistical Methods

For both indices employed, the mean value and standard error of each pathological subset were measured. Differences were tested for significance by analysis of variance, with subgroup analysis by Newmann-Keuls test (Wiener, 1971).

Table 1

<table>
<thead>
<tr>
<th>Subset</th>
<th>Value of Integrated Attenuation Index (per cm)</th>
<th>Comparison</th>
</tr>
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<tbody>
<tr>
<td>NL</td>
<td>24 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>FI</td>
<td>32 ± 3.1</td>
<td>*</td>
</tr>
<tr>
<td>FA</td>
<td>82 ± 6.5</td>
<td>*</td>
</tr>
<tr>
<td>CL</td>
<td>185 ± 8.7</td>
<td></td>
</tr>
</tbody>
</table>

NS = not significant. NL = normal arterial wall tissue, FI = fibrous arterial wall tissue, FA = fibrofatty arterial wall tissue, CL = calcified specimen.

\(\*P < 0.01\).

Results

Changes in Ultrasonic Integrated Attenuation Index Associated with Atherosclerosis

Figure 2 illustrates the relative values of the integrated attenuation index for normal and atherosclerotic arterial walls; a statistically significant difference is obtained between fibrous, fibrofatty, and calcified specimens (Table 1).

In Figure 3, a complete ultrasonic matrix (1 pixel = 1 acquisition) is shown for a specimen of each subset, together with a typical histological appearance. A pictorial tridimensional representation (256 levels) is adopted for the matrix display. Normal and pathological specimens are different, both for the absolute height value and, also, for the matrix pattern; i.e., normal specimens have points in the matrix very similar in intensity among them, whereas pathological specimens, on the contrary, show a highly inhomogeneous pattern.

In Figures 4 and 5, the best fit straight line for the \(\ln |H(f)|\) is shown for each of the 16 discrete sites of two specimens [one mostly fibrous (Figs. 4a and 5a), the other mostly fibrofatty (Figs. 4b and 5b)]. It is apparent that both the absolute value and the slope of the best fit straight line help to differentiate fibrous from fibrofatty samples.
Changes in Slope Index Associated with Atherosclerosis

Figure 6 illustrates the value of the slope index in the four subsets of normal and pathological specimens. This index can also differentiate between normal and fibrous specimens; however, there is a substantial overlap between fibrofatty and calcified samples (Table 2).

In Figure 7, the complete ultrasonic matrix (16 points) of four specimens (one for each subset) is shown.

Discussion

The results of this study indicate that alterations in ultrasonic attenuation may characterize the different pathological patterns of atherosclerosis in the arterial wall, providing a distinction between normal specimens and fibrous, fibrofatty, calcified plaques. Such alterations in ultrasonic attenuation may reflect intrinsic changes in biochemical and mechanical properties of atherosclerotic tissue.

The biochemistry of the atheroma documents an increase in at least three structural components potentially responsible for increased attenuation of the plaque: collagen, lipids, and calcium.

Fibrous plaques show a slightly higher attenuation, but in a nonstatistically significant way, compared with normal aortic walls. This finding is in agreement with the well-known fact that collagen is an important determinant of acoustic attenuation in soft tissues (Fields and Dunn, 1973; O’Donnel et al., 1979). However, the slope index—which is relatively independent from the angle of incidence of interrogating beam with the tissue, and reflects only the internal contribution of the sample to attenuation—has lower values in the fibrous than in the normal subset. This implies that a relevant compo-
FIGURE 5. Panel A: histological appearance (Weigert-Gieson, 16X) for each of the 16 sites ultrasonically studied and displayed in Figure 4A. Panel B: histological appearance (Weigert-Van Gieson, 16X) for each of the 16 sites ultrasonically studied and displayed in Figure 4B. Most sites are "fibrofatty," with different relative components of fibrous (f) and lipoidal (I) tissue. Two sites (panel B, no. 5 and no. 15) are completely normal; they have the lower absolute value in attenuation among the 16 sites, and also a less steep slope (see Fig. 6). The corresponding ultrasonic measurement at each site is shown in Figure 4B.

component of the overall attenuation measured by integrated attenuation index is due to surface (as opposed to internal) contribution of the sample. This surface contribution may recognize a morphological substrate in the fact that atherosclerosis (particularly in the uncomplicated phase of fibrous plaque) is primarily a disease of the intima (the first reflecting interface of the sample, which determines the surface contribution to attenuation). Only in its more advanced stages (fibrofatty and calcified samples), the atherosclerotic process involves the deeper layers of the wall and is therefore detectable with a significant increase in the slope index, which is sensitive only to internal contributions.

The increased attenuation in fibrofatty samples, compared to fibrous ones, could seem surprising, at least at first sight. Adipose tissue has a very low attenuation value among various tissues of the body, higher only than blood (Fields and Dunn, 1973). In other situations where pathology is characterized by a change in relative proportions of fibrous and adipose tissue (as in breast pathology), the increase in relative amount of fibrous tissue is known to determine an overall increase in attenuation (Kobayashi, 1979). In the atherosclerotic plaque, on the contrary, the increase in fat component raises the attenuation, also with a distinctly peculiar pattern of the slope.

A possible explanation could be that—in the plaque—the increase in fat component is also determined by cholesterol crystals [the high reflectivity of which was documented (Glancy et al., 1980)] and not by amorphous adipose tissue only. A significant deposition of cholesterol crystals was indeed seen by histology in 53% of fibrofatty samples. Another possible explanation could be that, in fibrofatty plaques, an acoustic interface is present inside the arterial wall, due to the impedance step between the fibrous cap and the lipidic core. Both such additional
interface and the accumulation of cholesterol crystals could lead to an increase in the reradiative loss of the signal, with a resulting increased attenuation.

As compared to fibrofatty specimens, a further increase in integrated attenuation index takes place in calcified specimens. This is not surprising, since calcium salts have by far the highest impedance among biological materials (Fields and Dunn, 1973).

Phase Cancellation Problems

Our measurements were made with phase-sensitive transducers. Phase cancellation effects due to the inhomogeneous nature of biological samples are a well-known potential source of error. The optimal solution to overcome such problems would be the use of a phase-insensitive acoustoelectric receiver (Busse and Miller, 1981); however, the use of a focused broadband transducer with a small diameter can substantially minimize phase cancellation artifacts, providing an adequate approach for studies in vitro. In fact, in a study performed in our laboratory (Landini et al., in press), phase cancellation effects [measured by means of statistical indexes, following the method of Mimbs et al. (1977)] were demonstrated to be: (1) minimized by employing focused small diameter transducers, (2) less evident with the 0.5 cm than with the 1.2 cm diameter focused transducer, and (3) less important in normal than in pathological specimens.

The recorded inhomogeneity of the matrix pattern, apparently more pronounced in pathological specimens as compared to normal ones (Figs. 3 and 9), has two possible explanations, one biological, the other methodological. This highly inhomogeneous pattern may reflect the spotty nature of the disease, whose only constant feature, from the pathologist’s point of view, is variability (McMillan, 1982). For instance, even in the same fibrofatty plaque, the relative amounts of fibrosis and fat deposition can vary from point to point (Fig. 5b), and histology is unable to quantify properly such subtle gradations.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Slope Values in the Four Subsets</th>
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<tr>
<td></td>
<td>Comparison</td>
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<tr>
<td></td>
<td>S (per cm per MHz)</td>
</tr>
<tr>
<td>NL</td>
<td>(10⁻⁹) 99.5 ± 9.1</td>
</tr>
<tr>
<td>FI</td>
<td>(10⁻⁹) 31.9 ± 4.5</td>
</tr>
<tr>
<td>FA</td>
<td>(10⁻⁹) 383 ± 21</td>
</tr>
<tr>
<td>CL</td>
<td>(10⁻⁹) 320 ± 23</td>
</tr>
</tbody>
</table>

NS = not significant.
* P < 0.05.
† P < 0.01.
The other possible explanation is that phase cancelation artifacts, due to structural variations inside the tissue, are most prominent in pathological specimens (Landini et al., in press) where internal inhomogeneities should be, on the average, more pronounced than in normal aortic walls.

Conclusions

Results of the present study demonstrate that it is possible to measure ultrasonic attenuation indices which characterize different types of atherosclerotic material. Even if ultrasound attenuation is not suitable, per se, for in vivo application, identification of attenuation properties of normal and diseased arterial tissue can be helpful in many ways. First, more basic data than those currently available are needed for a better understanding of the interaction between ultrasound and arterial tissue: this is a prerequisite for any development in the direction of ultrasonic characterization of atherosclerosis. Furthermore, there is the possibility that additional developments (e.g., measurements of ultrasonic attenuation based on backscattered signals) ultimately may permit the use in vivo of the approach employed (Flax et al., 1983). Of course, the feasibility of these various methods in the case of atherosclerosis remains to be evaluated, in a way similar to that done for attenuation in myocardium (Johnston et al., 1982).

It may be possible to utilize attenuation measurements as an experimental, or potentially clinical, tool, to characterize the nature of atherosclerotic plaque in the noninvasive, nondestructive fashion.

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