Quantitative Ultrasonic Characterization of Lesion Composition and Remodeling in Atherosclerotic Rabbit Aorta

Samuel A. Wickline, Richard K. Shepard, Alan Daugherty

We have previously shown that high-frequency, high-resolution ultrasound can characterize the acoustic properties and composition of fatty plaques in cholesterol-fed rabbits. To determine whether quantitative ultrasound can delineate the regression of atherosclerotic lesions by detecting a change in their composition from fatty to fibrous types induced by alterations in dietary regimen, we fed six New Zealand White rabbits a 2% cholesterol diet for 3 months, followed by a standard diet for 3 additional months to promote the development of fibrous intimal lesions. Segments of aortas were excised, and backscattered radiofrequency data were acquired from 400 to 600 independent sites in each specimen with an acoustic microscope operated at 50 MHz. Control data were provided by measuring backscatter from adjacent portions of the aortas devoid of lesions. Histological and immunocytochemical analyses of the fibrous intimal lesions confirmed the presence of smooth muscle cells and abundant connective tissue with little appreciable lipid. Backscatter from normal aortic segments (−30.7±1.0 dB) was approximately 10-fold greater than that from fibrous lesions (−42.4±1.0 dB; P<.05). We previously reported that integrated backscatter from fatty lesions was −50.6±0.7 dB, or approximately 10-fold less than that from fibrous lesions (P<.05). Values for integrated backscatter from the media of each tissue type were approximately equal (−30.0±1.7 versus −30.7±1.6 versus −33.4±0.8 dB for normal versus fibrous versus fatty tissues, respectively; P=not significant). Thus, quantitative ultrasonic tissue characterization sensitively differentiates between fibrous and fatty atherosclerotic plaque and normal tissue in the presence of experimental diet-induced atherosclerosis. These results suggest that alterations in dietary regimens may elicit changes in the composition of vascular plaques that are detectable with quantitative ultrasound. (Arterioscler Thromb. 1993;13:1543-1550.)

KEY WORDS: ultrasonic tissue characterization • acoustic microscopy • integrated backscatter • atherosclerosis • cholesterol-fed rabbits • cholesterol • plaque

Ultrasound imaging has emerged recently as a promising diagnostic modality for the delineation of the extent of atherosclerosis in peripheral and coronary arteries. Reliable measurements of vessel luminal diameter and wall dimension can be obtained with vascular ultrasound. Quantitative ultrasonic characterization of the biophysical composition of plaques in vivo has not been reported to date because the current generation of devices available for intravascular imaging permits only qualitative assessment of the acoustic characteristics of lesions. To our knowledge, no quantitative ultrasonic data exist regarding the scattering behavior of atherosclerotic lesions in vivo, and many of the available studies in vitro were performed at frequencies too low to be useful for assessment of coronary artery lesions.

One approach to quantitative vascular tissue characterization is to define the intrinsic scattering behavior of isolated components of normal and atherosclerotic vessels. We have previously shown that high-frequency, high-resolution ultrasound can be used to define the acoustic properties of fatty plaque in cholesterol-fed rabbits according to the measurement of integrated backscatter. Integrated backscatter is an objective index of tissue scattering referenced to the scattering from a near-perfect reflector, which provides a measure of acoustic properties independent of system electronics and transducer characteristics. These studies show that fatty intimal lesions manifest approximately 20 dB less integrated backscatter than does the media of either normal or atherosclerotic aortas.

The use of quantitative ultrasonic methods to delineate plaque components could have major clinical implications for defining the regression and the change in composition of atherosclerotic lesions in response to therapy with cholesterol-lowering agents or dietary regimens. Regression of fatty lesions can be induced with either oral probucol therapy or after discontinuation of a high-cholesterol diet. Both of these interventions promote an evolution from fatty to fibrous plaques after several months. The use of ultrasound to detect this transition in lesion composition has not been previously reported.

Accordingly, high-frequency (50 MHz), high-resolution acoustic microscopy was used to acquire radiofrequency (rf) data from segments of aortas excited from...
cholesterol-fed rabbits that had undergone lesion regression after cessation of the high-fat diet to produce primarily fibrous plaques. These data were compared with previous measurements of integrated backscatter from fatty lesions in rabbits that were acquired in the same manner as were the present data. Histochemical and immunocytochemical studies (Fig 1) were performed to verify the differences in composition between the fatty and fibrous lesions, which depend on the specific feeding regimen adopted. The results indicated that the change in composition of lesions induced by dietary therapy can be quantified by measurement of integrated backscatter.

**Methods**

**Experimental Preparation**

Six New Zealand White rabbits were fed a 2% cholesterol diet (120 g/24 h; Purina Mills) for 3 months.
Acquisition and Analysis of Ultrasonic Data

A custom-designed, high-resolution, high-frequency acoustic microscope was used to acquire backscattered rf data as described previously. Briefly, the acoustic microscope consisted of a 50-MHz, broadband, focused, piezoelectric delay-line transducer (1/4-inch diameter, 1/2-inch focal length; model V390, Panametrics Co, Waltham, Mass) operated in the pulse-echo mode with custom-designed electronics (General Electric Co, Schenectady, NY); an Aerotech Unidex 12 controller and servomotors that permit motion of the transducer in x, y, and z coordinates with 1-μm step resolution; a Tektronix DSA 601 digitizing oscilloscope (Beaverton, Ore); and a MacIntosh IIIfx computer with a GPIB interface bus to control the system and collect data. The propagated rf pulse envelope incorporated approximately 1.5 cycles with a duration of about 50 nanoseconds. The transducer was mounted on a micromanipulator that the focal zone was located in the middle of the arterial specimen.

Two scanning protocols were used to evaluate each arterial specimen. First, each specimen of aorta was imaged in a low-resolution C-scan mode to define the lateral boundaries of the tissue segments by moving the transducer in the x-y plane in 50-μm translational steps over the entire surface of the tissue and recording gated, peak-detected, digitized (8 bit) values for backscattered rf from regions just below the endothelial surface. The low-resolution C-scans were displayed on the computer monitor to identify regions of atherosclerosis denoted by raised intimal plaques. Backscattered rf data were recorded through the full thickness of the arterial specimen across the middle of the lesion. The rf data were recorded at independent sites evenly spaced by 100 μm along a single line across the full extent of the lesion. At each site, the received rf signals were digitized at 400 megasamples/s with 12-bit resolution. The rf data were averaged online 256 times by the DSA 601 oscilloscope to improve the signal-to-noise ratio. B-scanned images of the tissue were produced by full-wave rectification of the rf data. Fig 2 (top) illustrates a typical B-scan from an intact artery with a fibrous atherosclerotic plaque (thickened fibrous intima and relatively normal media). The figure has been color coded to enhance the different layers of the vessel, which would be more difficult to discern by visual inspection of gray-scale formatted images. The bottom portion of Fig 2 shows one of the rf lines used to construct the B-scan.

The B-scan images from the dissected intimal or medial segments were displayed on the monitor to select regions for analysis of integrated backscatter that avoided inclusion of specular echos. Segments of rf lines 400 nanoseconds in duration that fit within the intimal or medial boundaries displayed on the B-scans were gated out across the full width of each B-scan to include the entire scanned area. Power spectra of the gated data were determined by fast-Fourier transformation after multiplication by a Hamming window. The power spectra from tissue segments were referenced to the power spectrum backscattered from a near-perfect steel plate reflector to compute the frequency-dependent backscatter transfer function according to methods previously described. Integrated backscatter was computed from the average of the frequency-dependent backscat-
ter transfer function over the useful bandwidth of the transducer (approximately 30 to 55 MHz) and was expressed in decibels relative to scattering from the steel plate.

**Histological and Immunocytochemical Analyses**

Representative tissue specimens were sectioned from the midpoints of lesions where rf data had been recorded. The tissue was embedded in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin, Masson's trichrome, and van Gieson's elastin stains. Slides were viewed with a Nikon Optiphot microscope and photographed with a Nikon 8004 camera.

Contiguous 5-μm sections of aorta were cut from the same paraffin blocks of one typical specimen that had been processed for standard histological analysis, deparaaffinized with linolene xylene (3:1), rehydrated, and then etched with hydrogen peroxide. Immunocytochemistry was performed with commercially available kits obtained from Biomeda Corp, Foster City, Calif. Immunostaining of the fibrous plaques with monoclonal antibodies HHF-35, a smooth muscle-specific antibody, and RAM-11, a macrophage-specific antibody, was performed as previously described to delineate the cellular composition of normal and abnormal intima and media. Control data were provided by positive staining of other rabbit fatty (foam cell) plaques and normal medial smooth muscle cells with RAM-11 and HHF-35, respectively.

**Statistics**

The measurements of integrated backscatter from individual rf lines (generally 400 to 600 per aortic plaque) were combined to yield single average values of integrated backscatter for each rabbit for either fibrous lesions or for interspersed normal arterial regions. This method of analysis was used to provide a conservative estimate of the significance of differences among lesions based on the response to a selected feeding regimen. Analysis of variance (ANOVA) was used to test the significance of differences of integrated backscatter between normal regions and those of fibrous and fatty plaques. Intergroup comparisons were made with the Scheffé F test. Differences were deemed significant at the P<.05 level. The mean±SEM of the average measured values are reported in the text and figures.

**Results**

**Histology and Immunocytochemistry**

Four of the rabbits fed a high-cholesterol diet developed patchy raised lesions amid more normal-appearing endothelium. Two rabbits had no gross evidence of atherosclerotic plaque, despite adequate intake of the high-cholesterol diet. We have observed that a small number of rabbits that exhibit high cholesterol levels (>1000 mg/dL) after this feeding regimen may not develop atherosclerotic aortic plaque. Their aortas appear normal both histologically and by ultrasonic tissue characterization. The histological characteristics of the interspersed normal aortic segments from rabbits fed the high-cholesterol diet are similar to those previously described with a thin intima, uniform elastic and smooth cell layers in the media, and a loose adventitia.

Fig 1A illustrates a Masson's trichrome stain of the plaques, which reveals extensive deposits of connective tissue and a predominance of smooth muscle cells, particularly within the cap and at the periphery of the lesions. Marked intimal thickening was observed and the media was relatively normal. The thickness of these fibrous plaques in general was approximately the same as that of the previously studied fatty lesions. The fibrous intimal plaques manifested layers of loosely distributed connective tissue with smooth muscle cells interspersed.

Fig 1B is a van Gieson's stain that demonstrates a modest amount of elastin in the fibrous intima, primarily at sites where smooth muscle cells were more abundant, such as at the borders of the lesions or within the fibrous cap. The media displayed features typical of an elastic artery with interdigitated layers of smooth muscle, collagen, and elastin. The media of fibrous plaques also appeared similar to that of normal aortas. Rarely, islands of medial necrosis were observed (not shown).

Fig 1C demonstrates a fibrous lesion stained with HHF-35, which is immunoreactive with smooth muscle cell actins. It shows that the fibrous plaques are characterized by marked infiltration of smooth muscle cells into the intima, particularly at the edges of the lesions and within the cap. The media exhibited the expected preponderance of smooth muscle cells.

**Quantitative Ultrasonic Characterization of Fibrous Plaques**

Fig 3 shows a typical ultrasonic B-scan and an rf line from a normal segment of the aorta from one of the cholesterol-fed rabbits. The scan is color coded to facilitate illustration of the different vessel layers, with red representing higher backscatter and dark blue less. The normal segment of aorta shows prominent and uniform backscatter throughout the vessel wall, with no clear distinction between the thin layer of intima and the media, as described by Shepard et al. The unprocessed rf data also reveal no clear distinction between intima and media.

Fig 2 (top) shows a typical ultrasonic B-scan color coded to facilitate illustration of the different layers and an rf line from a fibrous plaque. The color scale is the same as that applied in Fig 3. The grossly thickened intima appeared as a dark blue band, indicating relatively less backscatter from the intima than the media. The lesions could be identified readily from inspection of their characteristic rf lines. The unprocessed rf line exhibited a specular echo at the tissue-water interface and lower scattering from the intimal plaque than from the media. Comparison of the ultrasonic B-scan in Fig 2 to the Masson's stain in Fig 1 demonstrates that the initial region of lower scattering emanated from the fibrous plaque and that the higher scattering emanated from the elastic media. Thus, the extent of intimal
Tissue Type

**FIG 4.** Graph of values of integrated backscatter from intima and media of fibrous plaques and from interspersed normal regions of atherosclerotic aortas from four rabbits. Integrated backscatter from the plaque intima differed significantly from that from the plaque media or normal regions.

Thickening and its distinctive scattering characteristics were apparent both from the B-scanned images and from individual rf lines.

Fig 4 compares the measurements of integrated backscatter from the fibrous plaques and the interspersed normal regions of the same atherosclerotic aortas from four rabbits. The fibrous atherosclerotic aortas exhibited substantially less integrated backscatter from the thickened intima than from subjacent media or their normal segments uninvolved with atherosclerotic plaque (−42.4±1.0 versus −30.7±1.5 versus −30.7±1.0, plaque intima versus plaque media versus normal regions, respectively; P<.05 by ANOVA). This difference represents an approximately 10-fold arithmetic reduction in integrated backscatter from the intima of fibrous plaques compared with either plaque or normal medial layers (P<.05 for intima versus either media).

**Discussion**

These data demonstrate that high-frequency, high-resolution ultrasonic examination of discrete vascular layers in aortas of cholesterol-fed rabbits can differentiate between predominantly fibrous atherosclerotic lesions and normal tissue in experimental diet-induced atherosclerosis. Quantitative characterization of fundamental scattering properties of normal and abnormal vessel layers can be determined by computing the integrated backscatter from segments of rf gated from within individual vessel layers.

**Comparison of Fatty and Fibrous Lesions**

We have previously shown that quantitative ultrasonic measurements of integrated backscatter can be used to characterize the composition of fatty lesions induced in rabbits by cholesterol feeding for 3 months. The present data indicated that there are substantial differences in the acoustic characteristics of fibrous versus fatty lesions in this model of diet-induced arteriopathy. Fig 5 illustrates the histological and ultrasonic characteristics of the normal and fibrous lesions compared with the previously studied fatty lesions. The fatty intimas comprise primarily foam cell infiltrates, whereas the fibrous lesions are primarily connective tissue and smooth muscle cells. Inspection of the unprocessed rf data reveals a significant difference in backscatter from the intimas of fatty and fibrous lesions. The medias appear to manifest equivalent backscatter.

The Table and Fig 6 compare quantitative data from this and the previous study. The integrated backscatter from intimal fatty plaques is approximately 10-fold less.

**Comparison of Average Integrated Backscatter From Different Types of Rabbit Aortic Tissue**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Integrated Backscatter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty plaque intima</td>
<td>4</td>
<td>-50.6±0.8*</td>
</tr>
<tr>
<td>Fibrous plaque intima</td>
<td>4</td>
<td>-42.4±1.0†</td>
</tr>
<tr>
<td>Fatty plaque media</td>
<td>4</td>
<td>-33.4±0.2‡</td>
</tr>
<tr>
<td>Fibrous plaque media</td>
<td>4</td>
<td>-30.7±1.6$</td>
</tr>
<tr>
<td>Normal media</td>
<td>4</td>
<td>-30.7±1.0‡</td>
</tr>
<tr>
<td>Control media</td>
<td>4</td>
<td>-28.0±2.9$</td>
</tr>
</tbody>
</table>

*P<.05 for fatty plaque intima vs fibrous plaque intima and vs all media segments.
†P<.05 for fibrous plaque intima vs all media/control segments.
‡P=NS for comparison among media/control segments.
observed in fibrous plaques, which manifest a less-smooth muscle cells, whereas lower backscatter was present of highly organized elastin, collagen, and scattering in the normal media was correlated with the greater amount of provided insights into the scatterers responsible for ob-

The validity of the comparison between these different groups of cholesterol-fed rabbits is supported by the data in the Table. Values for integrated backscatter from the medial regions of fatty and fibrous plaques, from interspersed normal regions in cholesterol-fed rabbits, and from normal vessel media layers in control animals all clustered around -30 dB (P=not significant for comparisons of backscatter among all medial/control layers). The methods used to measure integrated backscatter were identical in the present and the previous study except that the fibrous lesions were dissected to separate the intima from the media, whereas the fatty lesions were measured intact. This modification was necessary because of excess attenuation of ultrasound through the fibrous compared with the fatty intimas (unpublished data). Because we did not compensate explicitly for ultrasonic attenuation through the intimas in either data set, this change was implemented to avoid spuriously low values for backscatter from the media of fibrous lesions compared with fatty lesions. The uniformity of integrated backscatter values from all media in the two different studies confirms that our present and previous measurements are comparable and justifies the grouping of these data for comparison of the scattering from fatty and fibrous lesions. Thus, dietary manipulations in rabbits do not appear to significantly alter the ultrasonic characteristics of the media, but they do elicit abnormal scattering from the intima according to the specific dietary regimen adopted.

Histological and immunocytochemical analyses provided insights into the scatterers responsible for observed differences in integrated backscatter from atherosclerotic intima and media. The greater amount of scattering in the normal media was correlated with the presence of highly organized elastin, collagen, and smooth muscle cells, whereas lower backscatter was observed in fibrous plaques, which manifest a less-organized matrix of connective tissue and smooth muscle cells. Lipid-laden foam cells in the fatty plaques were associated with the least scattering.

These data did not indicate whether the composition of the plaques or the specific organization of their constituents is responsible for the observed differences in scattering behavior. Collagen has been shown to be a principal determinant of scattering in myocardial tissue. However, the specific size, shape, and three-dimensional organization of cardiac myocytes represent equally important parameters in the scattering equation. Anisotropy, or the angle dependence of scattering, also plays a role in the interaction of ultrasound with both cardiac and vascular tissue. Lipid-laden and fibrous plaques appear qualitatively less organized than does normal media in terms of the presence of a discrete elastic lamina. Unfortunately, the specific degree of organization of the two types of atherosclerotic plaque is difficult to quantify. Therefore, the relative contribution of plaque composition versus organization as potential mechanisms of scattering behavior remains an important topic for further investigation.

**Comparison With Other Studies**

Previous classifications of plaque constituents with ultrasound have relied primarily on qualitative interpretation of the image data to differentiate among fatty, fibrous, and calcific components. However, ultrasonic images reflect not only the physical characteristics of the tissue being studied but also the system gain and compression settings, lateral and axial resolution, beam frequency and bandwidth, beam diffraction, preprocessing and postprocessing schemes, tissue attenuation, and operator experience. These system features have not been standardized in clinical studies reported to date.

Quantitative tissue characterization with lower-frequency and lower-resolution ultrasonic systems have been reported. Barzilai et al examined human aortas with complex plaques with 10-MHz ultrasound and measured integrated backscatter within a 2-microsecond window (approximately 1.5-mm thickness of tissue). They report that arterial segments with extensive calcification demonstrated greater backscatter than did predominantly fibrofatty, fibrous, or normal aortas. Fibrous and fatty lesions were not well differentiated from each other or from normal tissue.

In contrast, our data from rabbit aortas indicated that relatively homogeneous fatty and fibrous lesions can be distinguished easily from each other and from normal elastic arterial tissue. The improved sensitivity in our study may have resulted from the use of a much shorter 400-nanosecond window for analysis of integrated backscatter (approximately 300 µm thickness of tissue in our case versus 1500 µm for Barzilai et al). The higher frequencies and greater resolution inherent in our measurements permit more selective examination of individual vessel layers than was possible in the study by Barzilai et al. This greater resolution is necessary to eliminate any potential confounding influence on scattering from other contiguous vessel layers or structures.

Picano et al also used 10-MHz ultrasound to compare backscattered rf data from human aortas with complex plaques that were assigned to different pathological classes based on qualitative histological analysis. They determined the amplitude of backscatter within a
potential clinical implications

One of the most promising methods for assessing plaque progression is to use quantitative ultrasonic tissue characterization. This approach can provide a rapid and cost-effective approach for quantifying the evolution of plaque composition induced by different dietary regimens. We did not compensate the values measured for integrated backscatter for the effects of ultrasonic attenuation. Nevertheless, we were able to easily differentiate fatty from fibrous lesions without accounting for this effect. Our approach mimics the clinical situation, in which attenuation would be difficult to measure directly, and therefore represents a feasible method for assessment of lesion characteristics.

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quantitative ultrasound. These tissue characterization methods should be applicable to clinical interrogation of atherosclerosis with high-frequency ultrasonic imaging devices.

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References

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