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Ultrasound molecular imaging of cardiovascular disease

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SUMMARY

Myocardial contrast echocardiography utilizes intravenously injected gas-filled microspheres as acoustically active red blood cell tracers. During ultrasound imaging, unimpeded microsphere transit through the intramyocardial microcirculation causes transient myocardial opacification, which can be mapped and quantified as myocardial perfusion. Ultrasound molecular imaging utilizes similar acoustically active microspheres, which are modified to bear a receptor-specific ligand on the surface, conferring microsphere binding to a disease- specific endothelial epitope. Because the microspheres adhere to the endothelium, ultrasound imaging reveals a persistent, rather than transient, contrast effect, indicating the presence and location of the molecule of interest in real time. Molecular contrast echocardiography has been developed to detect upregulated leukocyte adhesion molecules during microvascular inflammation, such as occurs in cardiac transplant rejection and ischemia–reperfusion. Principles of microsphere targeting and ultrasound imaging of microvascular epitopes have been extended to larger vessels to image molecular markers of atherosclerosis. This Article summarizes the current status of cardiovascular ultrasound molecular imaging. Experimental proofs of concept will be outlined and the clinical extension of these concepts to the molecular imaging of cardiovascular disease using clinical ultrasound technology will be discussed.

Keywords

adhesion molecules; atherosclerosis; contrast echocardiography; molecular imaging; ultrasound

INTRODUCTION

Molecular imaging with ultrasound offers the possibility of real-time, noninvasive visualization of molecular markers of cardiovascular disease using clinical ultrasound imaging systems. This technology utilizes microparticles or nanoparticles that are targeted to

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bind to function-specific epitopes after systemic injection. These bound particles (contrast agents) are acoustically active in the presence of ultrasound, resulting in a backscatter signal from the molecular event of interest, which can be localized and imaged by two-dimensional ultrasound scanning systems.

A growing body of literature on *in vitro* and animal models of disease substantiates the feasibility and clinical promise of ultrasound-based molecular imaging. This Article summarizes the current status of ultrasound imaging for the molecular identification of pathophysiologic states associated with various cardiovascular diseases. We review the status of targeted contrast agent development and the ultrasound imaging principles that form the basis for contrast detection. Reports demonstrating proof of concept for ultrasound molecular imaging in disease models are summarized and their extension to clinical applications is discussed.

PROPERTIES OF ULTRASONOGRAPHY CONTRAST AGENTS

Although a variety of ultrasound contrast agent formulations have been described,[1–6] all particles are micron to nano sized and are acoustically active in an ultrasound field. Our discussion focuses on the most studied gas-filled microspheres, so-called microbubbles, measuring 2–4 μm in diameter. Of these, various compositions have been reported and frequently include perfluorocarbon or nitrogen gas encapsulated by shells made of phospholipid, albumin, or biodegradable polymers.[1,2,6,7] Microbubbles are able to remain within the intravascular compartment because of their small size and, therefore, the molecular targets are necessarily endoluminal. Other ultrasound contrast agents that are not gas encapsulating microspheres are liposomes[8] and liquid perfluorocarbon emulsion nanoparticles that exit the intravascular space,[4,9] conferring the potential capacity for acoustically identifying extravascular targets, such as atherosclerotic plaque components.

Microbubble contrast agents are detectable because the frequencies used by ultrasound imaging systems cause the microbubbles to expand and contract.[10] At higher acoustic power settings, the microbubble oscillations become asymmetric, and at sufficiently high acoustic power, the microbubbles can be induced to rupture.[10] Such behaviors, termed nonlinear resonance, render the microbubbles strong ultrasound emitters themselves, resulting in distinct acoustic signals based on their unique frequency spectrum and profile.[11] Clinically available ultrasound machines can distinguish the nonlinear signals from tissue backscatter. The imaging systems vary with respect to incident ultrasound frequency, pulse waveform, acoustic power, whether micro-bubbles are induced to rupture, and detection frequency, details of which are reviewed elsewhere.[11] All system configurations display the processed microbubble signal as a video intensity increase on the two-dimensional image.

The use of microbubbles for molecular imaging is an extension of a pre-existing echocardiographic application that uses intravenously injected microbubbles, which have an intravascular rheology comparable to that of red blood cells,[12] to opacify the blood pool in order to delineate endocardial borders and allow more accurate assessment of left ventricular systolic function.[13] Two gas-filled microsphere ultrasound contrast agents are currently approved for this indication by the FDA.

Myocardial contrast echocardiography for tissue perfusion imaging further extends the concept of ultrasonic red blood cell tracking to the visualization of the blood volume in the intramyocardial microcirculation. Microvascular transit of the microbubbles during echocardiographic imaging causes a transient increase in myocardial tissue video intensity, which can be quantified and regionally mapped as myocardial perfusion. This approach has been applied to the detection of coronary artery disease[14, 15] and assessment of myocardial viability after acute myocardial infarction.[16, 17] Several commercial microbubble formulations for perfusion imaging are currently undergoing evaluation in multicenter clinical trials.[18]

The application of microbubbles for molecular imaging utilizes the properties described above, namely their intravascular location and acoustic responsiveness, to specifically probe molecular features of the endothelial surface. Unlike freely circulating microbubbles used for ventricular opacification and perfusion imaging, targeted microbubbles are designed to adhere to endothelium via specific ligand-receptor interactions that are prespecified in the microbubble design. A targeting ligand is attached to the surface of the microbubble, causing the microbubble to adhere to a specific endothelial marker. This adhesion manifests in the two-dimensional ultrasound image as a video intensity increase in the region where the molecular target is localized, and which persists even after circulating microbubbles have washed out (Figure 1).

The targeting ligands that were initially used to demonstrate the proof of concept were monoclonal antibodies directed against endothelial targets.[7,19,20] Other targeting moieties have since been evaluated, including peptide sequences[21–24] and naturally occurring ligands for the receptor being targeted, such as carbohydrates or proteins.[25–27] The adhesion of microbubbles to their targets is dependent on fluid shear stress levels at the vessel wall and directly proportional to receptor density on the endothelial cell.[28,29] Ligand density on the microbubbles, ligand– receptor bond affinity, and the conformational presentation of the ligand to the receptor are properties that affect net microbubble adhesion and thereby pose opportunities for optimization of microbubble design.[28,30]

PROOF OF CONCEPT FOR MOLECULAR IMAGING WITH ULTRASOUND

Microbubbles are intravascular tracers and, therefore, they are ideally suited for imaging endothelial inflammation, such as occurs during atherosclerosis, ischemia, and transplant rejection.[31] A microbubble-bearing monoclonal antibody to the leukocyte adhesion molecule, intercellular adhesion molecule-1 (ICAM-1), proved the concept that a gas-filled microsphere could adhere *in vitro* to a cultured biological surface expressing the antibody target.[7]

In vivo demonstrations of microbubble targeting and ultrasound detection of adhesion molecule overexpression have been subsequently reported.[19,20,25] Lindner and colleagues[19] imaged a microbubble bearing surface antibodies to P-selectin, after intravenous injection into mice with renal ischemia and/or reper-fusion. Postischemic kidneys demonstrated persistent contrast enhancement after injection of the targeted microbubbles, an effect that was not seen in normal kidneys or after injection of nontargeted microbubbles.

CLINICAL USE OF MICROVASCULAR ULTRASOUND MOLECULAR IMAGING

Leukocyte adhesion molecules are useful diagnostic targets due to their overexpression in both large arteries, as well as in the microcirculation in various cardiovascular disease states. For example, ultrasound detection of microvascular adhesion molecule overexpression can be applied to the clinical challenge of determining whether symptoms of acute or recent chest pain are due to myocardial ischemia or are noncardiac in origin, when typical diagnostic electrocardiographic features or cardiac enzymatic changes are not present. One approach to this diagnostic dilemma is to identify the so-called ischemic memory of myocardium by imaging an endothelial marker of acute or recent myocardial ischemia that persists even after the ischemic event has resolved. We have reported such a strategy using a microbubble targeted to P-selectin, a leukocyte adhesion molecule overexpressed by the endothelial surface within minutes of ischemia and/or reperfusion.[25] Microbubbles bearing the tetrasaccharide, sialyl Lewis^X—the naturally occurring ligand for P-selectin—were intravenously administered to rats after brief myocardial ischemia/reperfusion injury. Ultrasound imaging demonstrated persistent contrast enhancement localized to the region of prior myocardial ischemia (Figure 2). These findings suggest that transthoracic ultrasound imaging utilizing microbubbles that bind to a micro-vascular endothelial marker of prior ischemia may be useful not only in the etiologic diagnosis of patients presenting with acute or recent chest pain, but also in delineating the extent of myocardium at risk.

Transthoracic echocardiography combined with microbubbles targeted to bind to ICAM-1 might enable the noninvasive detection of acute heart transplant rejection, an inflammatory state associated with overexpression of leukocyte adhesion molecules. In a rat model of heterotopic cardiac transplantation with acute rejection, microbubbles bearing anti-ICAM-1 antibodies caused a persistent myocardial contrast effect not seen with nonsense-targeted microbubbles or with ICAM-1 targeted microbubbles in non-rejecting control myocardium.[20] Such data suggest that targeted contrast ultrasound, specifically routine two-dimensional echocardiography, could be useful in the noninvasive diagnosis of acute organ transplant rejection, thus precluding the requirement for invasive endomyocardial biopsy—the current gold standard for diagnosis of rejection.

Molecular echocardiography has shown promise for identifying angiogenesis. While the efficacy of therapeutic angiogenesis has been difficult to demonstrate in patients using traditional clinical metrics such as exercise tolerance and nuclear single-photon emission CT (SPECT) imaging,[32] ultrasound imaging of a molecular angiogenesis marker could provide a useful surrogate endpoint for a biological effect in patients undergoing therapeutic angiogenesis. Microbubbles targeted to $\alpha_v\beta_3$ integrins via peptides,[22–24] or to vascular endothelial growth factor (VEGF) receptors via the non-heparin binding isoform of VEGF, $VEGF_{121}$,[26] have been reported to identify angiogenic microcirculation in animal models of tumor-mediated or growth-factor-mediated angiogenesis. Such concepts can be extended to echocardiographic imaging for myocardial angiogenesis in response to therapeutic interventions, including genes or drugs.

ULTRASOUND MOLECULAR IMAGING OF ATHEROSCLEROTIC LESIONS

The imaging approaches described above were aimed at detecting molecular markers that are present in pathophysiologic processes affecting the microcirculation, such as postischemic reperfusion, transplant rejection, and angiogenesis. These approaches were based on an imaging paradigm developed for myocardial contrast echocardiography, which principally images the intramyocardial microvascular blood compartment.[33]

A strength of microbubble-based imaging is that the microbubbles remain within the intravascular space. In addition to permitting the molecular interrogation of the microvascular endothelium, this attribute opens possibilities for imaging molecular components of pathophysiologic events occurring in the endothelium of larger vessels, such as the inflammatory changes that initiate atherogenesis in the coronary or carotid arteries. Atherosclerosis is heralded by endothelial dysfunction, intimal xanthoma, and pathologic intimal thickening, a molecular hallmark of which includes the overexpression of adhesion molecules that mediate leukocyte infiltration of the endothelium.[31] As these molecules have already been shown to be ultra-sonically detectable with targeted microbubbles,[20,25] the strategies described above for microbubble design and imaging of micro-vascular adhesion molecules and angiogenic receptors can be extended to the preclinical detection of atherosclerotic plaques, atherosclerosis-prone endothelium in larger vessels, or both. The identification of leukocyte adhesion molecules on endothelium would be the basis for early disease detection (before luminal stenoses develop), and serve to mark ‘vulnerable patients’ who are at-risk for atherosclerotic cardiovascular disease even before clinical events, and hence are in need of primary preventative treatment.

Targeted ultrasound imaging of atherosclerosis presents two unique considerations not encountered in microvascular molecular imaging. First, in contradistinction to microvascular molecular imaging, in which microbubbles adhere within a matrix of intramyocardial microvessels imaged collectively in the ultrasound beam, the location of microbubble adhesion to an atherosclerotic plaque is more spatially confined, resulting in a diminished microbubble signal. Second, a critical component for microbubble detection is the appropriate coupling of the ultrasound imaging frequency with the natural resonance frequency of the microbubble, such that ‘harmonics’ can be elicited from the microbubble and detected as a signal distinct from tissue background. This resonance frequency, determined largely by microbubble size, is fortuitously the frequency used in standard clinical echocardiography (1–3 MHz). Vascular imaging, however, requires higher ultrasonic frequencies to achieve spatial resolution, such as that used for coronary intravascular ultrasound (IVUS; 30–40 MHz) or surface vascular ultrasound (7–12 MHz). Because this frequency range is not optimally matched to the natural resonance frequency of microbubbles sized 2–4 μm, the signal detection strategy for micro-bubbles adhered to the endothelium of larger vessels poses a challenge.

These issues notwithstanding, there have been promising developments in microbubble and imaging system design that raise hope for the ability of ultrasound molecular imaging to interrogate plaque components in larger vessels. Targeted echogenic lipid particles (termed immunoliposomes) coupled to antibodies directed against ICAM-1, vascular cell adhesion

molecule (VCAM)-1, fibrinogen, fibrin, and/or tissue factor increased ultrasound backscatter from atheromatous endothelium during IVUS imaging in a porcine model of atherosclerosis.[8] Due to the considerations cited above, however, the signal-to-noise ratio of this approach has been a limitation, as has the requirement for direct intra-arterial injection to achieve adequate particle adhesion.

Recently, more robust acoustic detection of inflammation in early atherosclerosis has been demonstrated with nonlinear, high- frequency ultrasound imaging of a spectrum of early lesions experimentally generated in apolipoprotein-E-deficient and wild type mice fed normal or high-cholesterol diets.[34] Microscopically verified thoracic aortic adhesion of intravenously injected lipid micro-bubbles bearing antitibodies to VCAM-1 was paralleled by persistent ultrasound contrast enhancement of the thoracic aorta (Figure 3) using noninvasive ultrasound vascular imaging. Furthermore, the magnitude of video intensity change was related to the extent of plaque formation, suggesting that VCAM-1-targeted microbubbles can detect, as well as quantify, inflammation in early atherosclerosis.

CONCLUSIONS AND FUTURE DIRECTIONS

The data discussed above establish that targeted ultrasound contrast agents adhering to endothelial epitopes can be imaged with scanning systems that are already clinically available. Although the approaches to ultrasound molecular imaging were initially developed in models of microvascular disease, the extension of this technology to the interrogation of larger vessels appears feasible. Readily apparent clinical applications of myocardial microvascular ultrasound molecular imaging include the noninvasive detection of myocardial ischemic memory in patients presenting with chest pain of uncertain etiology, heart transplant rejection, and myocardial angiogenic responses to therapeutic angiogenic interventions. Because atherosclerosis is a systemic disease of the blood vessel wall, acoustic detection of adhesion molecules in larger peripheral vessels, such as the carotid arteries, would identify patients in whom atherogenesis has already commenced, and hence provide a peripheral ‘window’ into coronary atherosclerotic risk. The noninvasive nature of this technology, the lack of requirement of radioisotopes, and the portability and widespread availability of ultrasound machines compared to nuclear or magnetic scanners, render ultrasound molecular imaging clinically appealing.

Additional improvements would facilitate clinical translation. First, optimization of microbubble design and formulation to increase adhesion to the target should improve signal to noise ratio. Such an effort could include identification of appropriate endothelial targets (high expression) as well as targeting moieties which confer specific and sustained microbubble adhesion, and/or development of new nonbubble contrast agents[4,8] or modified microbubble formulations[35] that are acoustically active.

Second, imaging platforms for microbubble detection need to be optimized to detect relatively small numbers of adhered bubbles as acoustically unique compared to nonadhered bubbles and background tissue. The optimal imaging system would incorporate a strategy based on an understanding of the acoustic behavior of adhered microbubbles, which seems to be distinct from that of freely circulating bubbles.[36,37] This approach would require

systematic optical and acoustical characterization of varying microbubble formulations so that the microbubble detection strategy could be optimized to the specific microbubble. Such an approach, some efforts for which have already begun,[38] should result in more robust image signals from the specific target.

For vascular imaging, an additional challenge lies in the optimization of microbubble signal and detection in the high frequency range required for adequate spatial resolution. The use of acoustic radiation force to 'move' bubbles towards the target could enhance microbubble adhesion.[39] The design of microbubbles that resonate at higher frequencies might be possible by manipulating size and shell characteristics. In conjunction, higher frequency transducers can be designed to detect microbubble resonance at higher frequencies. For direct coronary ultrasound molecular imaging, invasive, catheter-based technology will be a necessary requirement. Recently, the design of a harmonic IVUS transducer has been reported which appears capable of detecting contrast-enhanced adventitial vasa vasorum in atherosclerotic rabbit abdominal aortas.[40] If this or other IVUS designs implemented to detect nonlinear microbubble behavior prove to be robust, and if microbubbles can be developed to bind to markers of plaque vulnerability, then there is even the exciting possibility of differentiating rupture risk, and hence customizing treatment, for nonocclusive plaques identified at the time of coronary angiography. The multidisciplinary efforts entrained in this effort thus far raise promise that this technology will come to clinical fruition in the not-too-distant future.

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KEY POINTS

- Ultrasound molecular imaging is based on the use of gas-filled microspheres engineered to bind to function-specific endothelial markers of disease via specific ligand-receptor interactions and that can be detected using clinical ultrasound scanning
- Targeted ultrasound imaging of leukocyte adhesion molecules can be used clinically to noninvasively identify myocardial ischemic memory, angiogenesis, and acute heart transplant rejection
- Catheter-based intravascular ultrasound imaging of adhesion molecules might permit identification of atherosclerosis-prone endothelium before luminal stenosis, facilitating early-stage diagnosis, and hence preventative treatment
- Optimization of microbubble and transducer design holds promise for the clinical translation of ultrasound molecular imaging to human populations in the foreseeable future

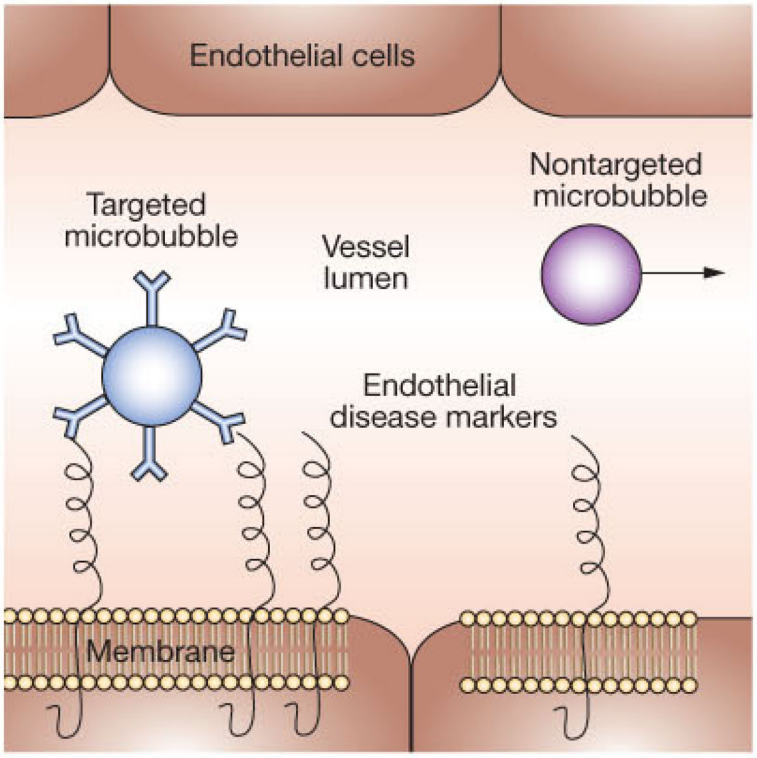

Figure 1.

Basis for molecular imaging with microbubbles. Schematic of a blood vessel with dysfunctional endothelium bearing surface markers of disease (e.g. inflammation). A targeted microbubble bears a ligand on its surface that confers specific binding to the disease marker, whereas a nontargeted microbubble has a nonreactive surface and transits through the circulation without adhering to the endothelium. Figure not drawn to scale.

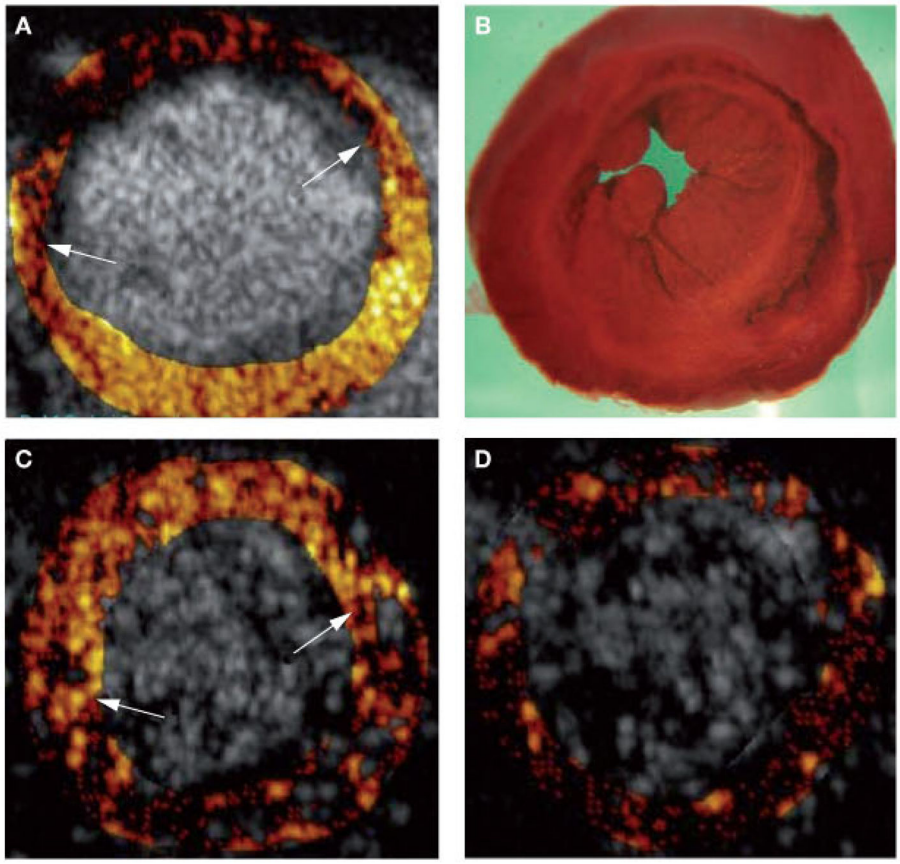

Figure 2.

Color-coded short axis ultrasound images and the corresponding myocardial specimen of the left ventricle demonstrating imaging of ischemic memory in a rat undergoing transient coronary artery occlusion. Gradations of red, to orange, yellow, and white represent increased contrast change. (**A**) During acute coronary occlusion, standard contrast perfusion imaging with nontargeted microbubbles demonstrates an area at risk for necrosis (upper region between arrows). (**B**) After successful reperfusion there is no infarction, as shown by post mortem staining with triphenyltetrazolium chloride in the myocardial specimen. (**C**) During the reperfusion period, ultrasound imaging after injection of microbubbles targeted to P-selectin shows persistent contrast enhancement in the previously ischemic region (upper region between arrows). (**D**) Contrast persistence in the risk area is not seen after injection of control nontargeted microbubbles conjugated to sialyl Lewis^{C}, a defucosylated molecule with less selectin affinity. Permission obtained from Lippincott, Williams & Wilkins © Villanueva FS *et al.* (2007) *Circulation* 115: 345–352.

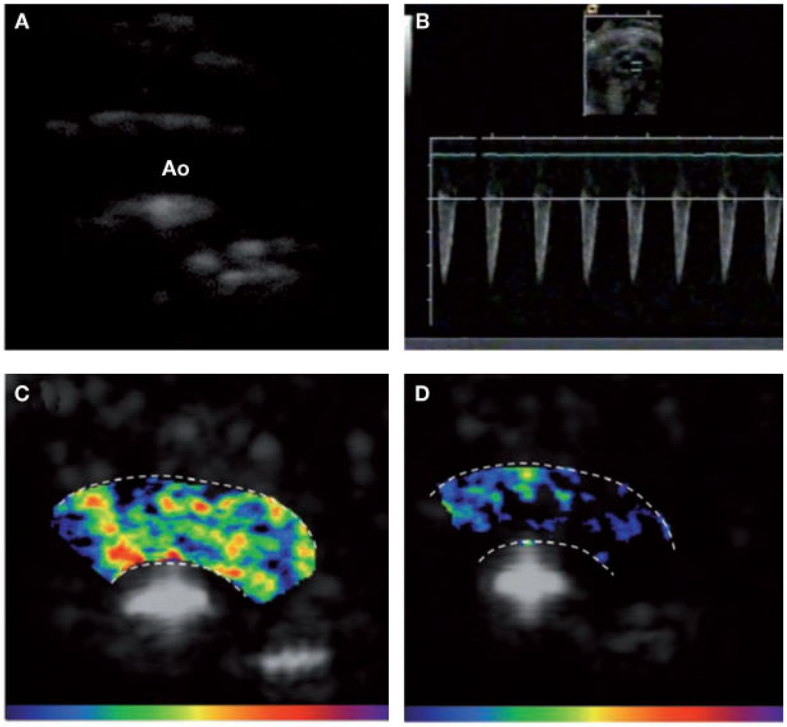

Figure 3.

Ultrasound molecular imaging of early atherosclerosis in atherosclerosis-prone mice *(ApoE* deficient) fed a high-cholesterol diet. (**A**) B-mode imaging of the aortic arch. (**B**) Spectral Doppler imaging of the aortic arch. (**C**) Ultrasound imaging 10 min after injection of microbubbles targeted to vascular cell adhesion molecule via an antibody directed against vascular cell adhesion molecule-1 demonstrates persistent contrast enhancement of the aortic arch. (**D**) Contrast persistence is not seen after injection of control microbubbles conjugated to an isotype control antibody. Permission obtained from Lippincott, Williams & Wilkins © Kaufmann BA *et al.* (2007) *Circulation* 116: 276–284. Abbreviation: Ao, aorta.