Molecular Imaging of Inflammation in Atherosclerosis With Targeted Ultrasound Detection of Vascular Cell Adhesion Molecule-1

Beat A. Kaufmann, MD; John M. Sanders, BS; Christopher Davis, MD; Aris Xie, MS; Patrick Aldred, MS; Ian J. Sarembock, MB, ChB; Jonathan R. Lindner, MD

Background—The ability to image vascular inflammatory responses may allow early diagnosis and treatment of atherosclerosis. We hypothesized that molecular imaging of vascular cell adhesion molecule-1 (VCAM-1) expression with contrast-enhanced ultrasound (CEU) could be used for this purpose.

Methods and Results—Attachment of VCAM-1–targeted and control microbubbles to cultured endothelial cells was assessed in a flow chamber at variable shear stress (0.5 to 12.0 dynes/cm²). Microbubble attachment to aortic plaque was determined by en face microscopy of the thoracic aorta 10 minutes after intravenous injection in wild-type or apolipoprotein E–deficient mice on either chow or hypercholesterolemic diet. CEU molecular imaging of the thoracic aorta 10 minutes after intravenous microbubble injection was performed for the same animal groups. VCAM-1–targeted but not control microbubbles attached to cultured endothelial cells, although firm attachment at the highest shear rates (8 to 12 dynes/cm²) occurred only in pulsatile flow conditions. Aortic attachment of microbubbles and targeted CEU signal was very low in control wild-type mice on chow diet. Aortic attachment of microbubbles and CEU signal for VCAM-1–targeted microbubbles differed between treatment groups according to extent of VCAM-1–positive plaque formation (median CEU videointensity, 1.8 [95% CI, 1.2 to 1.7], 3.7 [95% CI, 2.9 to 7.3], 6.8 [95% CI, 3.9 to 7.6], and 11.2 [95% CI, 8.5 to 16.0] for wild-type mice on chow and hypercholesterolemic diet and for apolipoprotein E–deficient mice on chow and hypercholesterolemic diet, respectively; \( P < 0.001 \)).

Conclusions—CEU molecular imaging of VCAM-1 is capable of rapidly quantifying vascular inflammatory changes that occur in different stages of atherosclerosis. This method may be potentially useful for early risk stratification according to inflammatory phenotype. (Circulation. 2007;116:276-284.)

Key Words: atherosclerosis ■ echocardiography ■ imaging ■ inflammation

Atherosclerosis is a chronic inflammatory disorder that often progresses silently for decades before becoming clinically evident.1 In current clinical practice, C-reactive peptide is the only inflammatory marker routinely used for risk assessment in patients. Noninvasive imaging of vascular changes such as coronary calcification, carotid intima-media thickening, and plaque morphology has recently been used to assess patient risk.2–6 However, these methods detect changes that occur relatively late in the disease process and do not directly assess inflammatory status. Because inflammation participates in plaque initiation and progression, a method capable of imaging the extent of vascular inflammation could potentially provide powerful predictive information on both early disease presence and future risk for disease progression. At latter stages of disease, it also could provide information on plaque vulnerability to erosion and rupture.7 It also is important to recognize that new therapies aimed at inhibiting vascular inflammatory responses are being developed and will likely be most effective when used in conjunction with quantitative methods that can detect early inflammatory changes.

Vascular cell adhesion molecule-1 (VCAM-1), expressed by activated endothelial cells, participates in leukocyte rolling and adhesion primarily by interacting with its counterligand VLA-4 (\( \alpha_4\beta_1 \)) on monocytes and lymphocytes.8,9 VCAM-1 expression on the vessel endothelial surface or the underlying vasa vasorum plays an important role in atherosclerotic plaque development by monocyte and T-lymphocyte recruitment.10 It is an ideal target for molecular imaging because there is little constitutive expression and its upregulation occurs at the earliest stages of atherogenesis.11,12 We hypothesized that molec-
ular imaging of VCAM-1 with targeted contrast-enhanced ultrasound (CEU) could be used to evaluate the degree of vascular inflammation in atherosclerosis. CEU is well suited for such screening purposes because of practical considerations such as cost, short duration of imaging protocols (10 minutes), and balance between spatial resolution and sensitivity for targeted contrast agent detection. To test our hypothesis, attachment of VCAM-1–targeted microbubbles to endothelial cells was evaluated under variable shear conditions. Microbubble attachment in vivo and signal enhancement of the aorta were assessed in animal models of various degrees of atherosclerosis produced by dietary intervention in wild-type and apolipoprotein E–deficient (ApoE−/−) mice.

**Methods**

**Microbubble Preparation**

Biotinylated, lipid-shelled decafluorobutane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of distearyloxyphosphatidylcholine, polyoxyethylene-40-stearate, and distearyloxyphosphatidylethanolamine-PEG(2000)biotin. Rat anti-mouse monoclonal IgG1 against VCAM-1 (MK 2.7) or isotype control antibody (R3–34, Pharmingen Inc, San Diego, Calif) was conjugated to the surface of microbubbles as previously described to produce VCAM-1–targeted (MBv) or control (MBc) microbubbles.13 For flow-chamber and in vivo attachment studies, microbubbles were fluorescently labeled by the addition of either dioctadecyltetramethylindocarbocyanine (DiI) or dioctadecylxocarbocyanine (DiO) perchlorate (Molecular Probes Inc, Carlsbad, Calif) to the aqueous suspension. Microbubble concentration was measured by electrozone sensing (Multisizer III, Beckman Coulter, Fullerton, Calif).

**Flow-Chamber Adhesion Studies**

Murine endothelial cells (SVEC4–10, ATCC, Manassas, Va) that express VCAM-1 were grown to confluence in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum on fibronectin-coated culture dishes.14 For activation, cells were pretreated with tumor necrosis factor-α (20 ng/mL) for 4 hours. Culture dishes were mounted on a parallel plate flow chamber (Glycotech, Gaithersburg, Md) with controlled gasket thickness and a channel width of 2.5 mm. The flow chamber was placed in an inverted position on a microscope (Axioskop2-FS, Carl Zeiss Inc, Thornburg, Calif) with a ×40 objective and high-resolution charge-coupled device camera (C2400, Hamamatsu Photonics, Hamamatsu City, Japan) for video recording. A suspension of control or VCAM-1–targeted microbubbles (3×10⁶ mL⁻¹) in cell culture medium was drawn through the flow chamber with a节发clude withdraw pump. The number of microbubbles attached to cells was determined for 20 optical fields (total area, 0.5 mm²) after 5 minutes of continuous flow at rates to generate shear rates of 0.5 to 12.0 dynes/cm². Experiments were performed in triplicate as a minimum.

Because aortic flow is pulsatile, adhesion at the highest shear rates (8 to 10 dynes/cm²) was measured through an anterior thoracotomy. The blood volume was removed with 10 mL of 5% bovine serum albumin containing heparin at 35°C to 37°C infused via a left ventricular puncture at an infusion pressure ≤100 mm Hg. The aorta was removed, a longitudinal incision was made, and the aorta was pinned flat on a microscopy platform. En face microscopy of the aortic arch and descending portions of the thoracic aorta was made with a ×20 objective. A minimum of 10 optical fields were observed under fluorescent epi-illumination at excitation wavelength lengths of both 490 and 530 nm.

**CEU Imaging**

Ultrasound imaging (Sequoia, Siemens Medical Systems, Mountain View, Calif) was performed with a high-frequency linear-array probe held in place by a raised gantry system. The aortic arch and proximal descending aorta were imaged from a left parasternal window with fundamental imaging at 14 MHz to optimize the imaging plane in the longitudinal axis. CEU was performed with Contrast Pulse Sequencing (Siemens), which detects the nonlinear fundamental signal component for microbubbles. Imaging was performed at a centerline frequency of 7 MHz and a mechanical index of 0.2. The gain was set just below visible speckle at baseline and held constant. Real-time imaging was performed 10 minutes after intravenous injection of 1×10⁶ MBv or MBc performed in random order. After several seconds of continuous imaging at a mechanical index of 0.2, microbubbles in the sector were destroyed by increasing the mechanical index to 1.0 for 1 second. Subsequent postdestruction images were acquired at a mechanical index of 0.2. To determine signal from retained microbubbles alone, several postdestruction contrast frames representing freely circulating microbubbles were averaged and digitally subtracted from several averaged predestruction frames.13 Background-subtracted intensity was measured from a region of interest placed over the aorta with the 14-MHz image as a guide.

Because microbubble attachment is dependent on contact with the aortic wall, the axial distribution of microbubbles immediately after injection was assessed in 3 wild-type mice. Imaging was performed with an ultrahigh-frequency (30 MHz) mechanical sector imaging system (Vevo 770, VisualSonics Inc, Toronto, Ontario, Canada) during an intravenous injection of MBc (1×10⁶). Ultrasound was transmitted with 1-cycle pulses with an axial resolution of 55 µm. Images were aligned and displayed as a maximum-intensity projection for 3 seconds after microbubble appearance.

**Measurement of Ultrasound Pressure Profile**

The 2-dimensional display of ultrasound reflects information that is received from a 3-dimensional beam that has an out-of-plane elevational dimension. Therefore, it was necessary to determine the portion of the mouse aorta (imaged in long axis) that fit within the detection profile of the elevational plane to determine whether contrast material from the front and back walls would be detected. Acoustic pressures within the imaging sector were measured in a water bath with a needle hydrophone (PVD-F-Z4, Specialty Engineering Associates, Sunnyvale, Calif) coupled with an oscilloscope (TDS-3012, Tektronix Inc, Beaverton, Ore). Peak negative acoustic pressure measurements were made at the focal depth using the system settings for targeted imaging. A 2-dimensional pressure profile was obtained by making 0.5-mm adjustments in the in-plane

**Animal Models and Preparation**

The study protocol was approved by the institutional Animal Research Committee. Twenty-six male wild-type C57Bl/6 and 23 ApoE−/− mice (The Jackson Laboratory, Bar Harbor, Maine) were studied at 22 to 24 weeks of age. Mice were fed either chow diet or, from 14 weeks of age on, a hypercholesterolemic diet (HCD) containing 21% fat by weight, 0.15% cholesterol, and 19.5% casein without sodium cholate. Anesthesia was induced with an intraperitoneal injection (12.5 µL · g⁻¹) of a solution containing ketamine hydrochloride (10 mg · mL⁻¹), xylazine (1 mg · mL⁻¹), and atropine (0.02 mg · mL⁻¹). A jugular vein was cannulated for administration of microbubbles.

**Assessment of Microbubble Attachment to the Aorta**

In anesthetized mice, VCAM-1–targeted and control microbubbles (1×10⁶ for each) labeled with DiI and DiO, respectively, were injected simultaneously by intravenous route. After 10 minutes, a right thoracotomy incision was made through the parasternal area. The flow chamber system was set to the minimum required for significant flow reduction as a result of the transmural pressure measurements were made at the focal depth using the system settings for targeted imaging. A 2-dimensional pressure profile was obtained by making 0.5-mm adjustments in the in-plane...
lateral dimension (beam width) and elevational dimension (beam thickness).

**Echocardiography**
The peak flow velocity at the midarch was measured by pulsed-wave Doppler with a gate size at the minimum setting. Left ventricular systolic function was assessed by imaging in the short-axis plane at the midpapillary muscle level with fundamental imaging at 14 MHz. Fractional shortening in the anterior-posterior and septal-lateral dimensions was measured by video calipers and averaged.

**Immunohistology**
Immunostaining for VCAM-1 was performed on paraffin-embedded sections of the proximal and distal aortic arch after microwave treatment with Antigen Unmasking Solution (Vector Laboratories, Burlingame, Calif) for several animals in each group. Goat polyclonal antibody to human VCAM-1 (sc1504, Santa Cruz Biotechnology Inc, Santa Cruz, Calif) was used as a primary antibody with a biotinylated secondary anti-goat antibody (Vector Laboratories). Staining was performed with a peroxidase kit (ABC Vectastain Elite, Vector Laboratories) and 3,3'-diaminobenzidine chromagen (Dako, Glostrup, Denmark). Slides were counterstained with hematoxylin.

**Statistical Analysis**
Unless otherwise specified, parametric data are expressed as mean±SD. Comparisons between microbubble agents for flow chamber studies were made by the Mann-Whitney rank sum test. Flow chamber data with sequential flow interruptions and targeted imaging data were analyzed with mixed-effect repeated-measures models. For the latter, microbubble preparation and animal group were included as fixed factors, as well as the interaction between the 2. Follow-up comparisons were made between bubble types for each animal group using a Bonferroni multiple-comparison adjustment. For microscopy data, individual comparisons were performed with 1-way ANOVA and a Tukey post hoc test or, when appropriate, with a Kruskal-Wallis test with Dunn post-hoc test. Differences were considered significant at P<0.05 (2 sided).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Microbubble Attachment to Endothelial Cells In Vitro**
Both nonactivated and activated cultured SVECs stained positive for VCAM-1 on immunohistochemistry. During flow-chamber studies at the lowest shear rate (0.5 dyne/cm²), there was minimal attachment of MBc to SVECs regardless of activation status (Figure 1A). MBc attached to both nonactivated and activated SVECs, with slightly more attachment to activated cells. Attachment of MBc to activated SVECs decreased with increasing shear rate (Figure 1B). Little microbubble attachment occurred at continuous shear rates that exceeded 6 dynes/cm². However, sequential brief reductions in shear allowed MBc to permanently bind at the highest shear rates tested (8 and 12 dynes/cm²) (Figure 1C), indicating the ability of microbubbles to firmly attach in the face of high shear when flow occurs in pulsatile rather than continuous conditions.

**Attachment of Microbubbles to the Aorta**
Ex vivo fluorescent microscopy of thoracic aortas removed 10 minutes after intravenous microbubble injection demonstrated little attachment for either control or VCAM-1–targeted microbubbles in wild-type mice on chow diet (Figure 2). In the other groups (wild-type mice on either chow or HCD), attachment of VCAM-1–targeted microbubbles to the aorta was greater than for control microbubbles. Attachment of VCAM-1–targeted microbubbles was significantly greater in ApoE−/− mice on HCD than in any other group and was distributed throughout the aorta. In contrast, in ApoE−/− mice on chow diet, VCAM-1–targeted microbubbles tended to attach preferentially to regions of the aorta where there was irregular thickening consistent with atherosclerotic lesion development.

**Targeted Imaging of VCAM-1 Expression**
There were no significant differences between groups in left ventricular fractional shortening, peak systolic flow velocity in the aorta, or aortic diameter at the midarch (the Table), indicating no systematic differences in hemodynamic conditions in the aortic arch. Flow velocities in the aortic arch decreased to 0 m/s at end diastole in most animals. CEU with ultra–high-frequency (30-MHz) maximum-intensity projection demonstrated that the axial distribution of nontargeted microbubbles during their transit through the aorta extended to regions directly adjacent to the aortic wall in both the greater and lesser curvature of the arch (Figure 3).

Illustrative B-mode, pulsed-wave Doppler, and background-subtracted color-coded CEU images from a...
Strong signal enhancement was observed for VCAM-1–targeted but not control microbubbles. The profile of the peak negative acoustic pressures at the acoustic focus for the transducer and settings used for targeted CEU are illustrated in Figure 5. According to the dimensions of the elevational plane, the entire volume of the aortic arch would be exposed to a peak negative acoustic pressure of \(120 \text{ kPa}\) before accounting for attenuation and \(96 \text{ kPa}\) after correcting for attenuation assuming a coefficient of \(1.1 \text{ dB} \cdot \text{mm}^{-1} \cdot \text{MHz}^{-1}\) (Figure 5). These data indicate that the entire circumference of the aorta (circle in Figure 5B) would fit in the effective detection profile of elevational plane. Hence, elevation plane averaging would permit detection of microbubbles attached to the front or back wall that were seemingly “out of plane” and explains the appearance of targeted stationary microbubble signal in the center of the apparent “lumen” in Figure 4. Figure 6 summarizes CEU data for all groups. Signal enhancement for control microbubbles was low and similar between groups. In wild-type mice on chow diet, signal for VCAM-1–targeted microbubbles was low and similar to that for control microbubbles. In contrast, in all other groups, there was greater signal enhancement for VCAM-1–targeted compared with control microbubbles. Signal enhancement for VCAM-1–targeted microbubbles increased incrementally from wild-type mice on HCD to ApoE\(^{-/-}\) mice on chow to ApoE\(^{-/-}\) mice on HCD. The interaction between microbubble agent and animal group was highly significant \((P<0.0001)\), suggesting that the difference in signal for control and VCAM-1–targeted microbubbles depended on group.

### Echocardiographic and Vascular Ultrasound Characteristics

<table>
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<th>Wild-Type Mice on Chow Diet ((n=9))</th>
<th>Wild-Type Mice on HCD ((n=10))</th>
<th>ApoE(^{-/-}) Mice on Chow Diet ((n=10))</th>
<th>ApoE(^{-/-}) Mice on HCD ((n=8))</th>
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<td>Aortic peak velocity, m/s</td>
<td>0.53±0.10</td>
<td>0.52±0.7</td>
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<td>Fractional shortening, %</td>
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<td>0.35±0.05</td>
<td>0.34±0.05</td>
<td>0.38±0.04</td>
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<tr>
<td>Aortic diameter, mm</td>
<td>1.2±0.2</td>
<td>1.4±0.1</td>
<td>1.3±0.2*</td>
<td>1.4±0.03</td>
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Figure 2. Microbubble attachment to the thoracic aorta 10 minutes after intravenous injection assessed by ex vivo fluorescent microscopy. A, Mean (±SEM) attachment of MB\(_B\) and MB\(_V\) microbubbles. \(^*P<0.05\) vs MB\(_B\); \(^\dagger P<0.05\) vs MB\(_B\) in wild-type mice on chow diet; \(^\ddagger P<0.05\) vs MB\(_B\) in all other groups. B, Examples of en face fluorescent microscopy of the thoracic aorta after injection of Dil-labeled MB\(_V\). Examples of the ApoE\(^{-/-}\) mouse on chow diet are shown for regions with and without evidence for irregular wall thickening on transillumination. Scale bar=1 mm.

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Figure 3. Distribution of nontargeted microbubbles in transit through the aortic lumen assessed by high-frequency (30-MHz) CEU acquired at a frame rate of 20 Hz. A, Illustrations of regions of interest spanning from position 1 (adjacent to the greater curvature) to position 5 (adjacent to the lesser curvature). B through H, Maximum intensity projection images 400 ms apart during appearance phase of microbubbles in the aorta demonstrating diffuse distribution of microbubbles throughout the lumen. The graph at the bottom depicts CEU maximum intensity projection data for the different regions of interest. Data were not significantly altered by analysis of frames only from end systole or end diastole.
Immunohistochemistry

On histology, there was no evidence for plaque development in wild-type mice regardless of diet. On immunostaining, however, VCAM-1 expression was detected on the luminal endothelial surface of the aorta in wild-type mice on HCD (Figure 7). In ApoE<sup>−/−</sup> mice, there was intimal thickening and large atherosclerotic plaques protruding into the lumen, particularly in animals on HCD. Immunohistochemistry in ApoE<sup>−/−</sup> mice demonstrated dense VCAM-1 expression on the endothelium, particularly overlying regions of plaque development. There also was VCAM-1 staining of neointimal monocytes. The degree of VCAM-1 staining on cells in the neointima qualitatively correlated with the degree of endothelial staining and was more robust in ApoE<sup>−/−</sup> mice when fed an HCD.

Discussion

The critical role that inflammation plays in atherosclerosis has produced a swell of interest in better methods to evaluate it. Ideally, such a technique should be specific for inflammatory responses that occur in the vasculature, sufficiently sensitive to detect early events, able to provide spatial information, and practical in terms of cost, speed, and ease of use for application as a rapid screening tool. To that end, we investigated whether CEU molecular imaging could be used to evaluate expression of the endothelial cell adhesion molecule VCAM-1 in murine models of atherosclerosis. VCAM-1–targeted signal enhancement in the different animal groups in this study varied according to the severity of atherosclerotic plaque development.

Molecular Imaging in Atherosclerosis

A method for imaging vascular inflammation may have a major impact in both the clinical and research laboratory settings. Strategies currently used to evaluate risk of cardiovascular disease or major adverse cardiac events may not necessarily meet the clinical needs of the future, given the trend toward earlier and more aggressive therapy. The Framingham risk score and modifications thereof take into account multiple different clinical variables. However, ≈40% of the adult US population fall into an intermediate-risk category<sup>16</sup> with a 6% to 20% risk of developing symptomatic coronary heart disease within the ensuing 10 years. Further refinement in risk stratification for this intermediate-risk category is desirable to make better use of long-term preventive therapies. There is also the notion that atherosclerosis, like many other diseases, is most amenable to treatment at an early stage. Efforts are underway to create novel therapies aimed at interrupting the inflammatory events that initiate plaque formation and trigger secondary growth responses. If treatment is to be initiated years to decades before atherosclerosis would otherwise become clinically evident, then a method for accurately detecting vascular inflammation would seem to be a critical factor.

Methods currently used to evaluate those who have developed symptoms of cardiovascular disease are designed to measure either the anatomic severity of disease or the physiological consequences of increased circuit
resistance such as ischemia or reduced flow reserve. Imaging the inflammatory phenotype in those patients will likely add unique information because inflammation is a key factor in the progression to unstable disease. The recruitment of inflammatory cells to the neointima results in release of prothrombotic, promitogenic, proangiogenic, and detrimental vasoactive molecules; release of oxygen-derived free radicals; and production of proteases that contribute to adverse remodeling and erosion of the plaque protective barrier. It is necessary that new methods for evaluating inflammation occur in parallel with new therapeutic strategies. Likewise, the use of molecular imaging in the preclinical development of therapies would provide a means to assess the pathogenic pathways being targeted. For this application, a technique should be quantitative, have high-throughput capacity, and possess sufficiently high-resolution for small animal model testing.

Microbubbles as a Targeting Agent

In this study, we chose to target microbubble contrast agents to VCAM-1. Microbubble contrast agents are pure intravascular agents and accordingly do not have access to extravascular events or epitopes that have been proposed for targeting such as resident inflammatory cells (macrophages, T lymphocytes), proteases, or oxidation byproducts. Instead, we targeted an endothelial cell adhesion molecule that is a critical participant in inflammatory cell recruitment in atherosclerosis. VCAM-1 is present on endothelial cells early during the development of atherosclerosis and is otherwise expressed only in very low levels. VCAM-1 has been investigated as a potential target for molecular imaging in mice with other imaging techniques such as targeted infrared

Figure 6. Background-subtracted CEU signal intensity from the aortic arch 10 minutes after intravenous injection of MB_{C} and MB_{V} microbubbles in the different animal groups. Data depict median value (horizontal line), 25% to 75% percentiles (box), and range of values (whiskers). The interaction between microbubble agent and animal group by mixed-effect repeated-measures analysis was significant (P<0.0001). *P<0.05 vs MB_{V} in wild-type mice on chow diet; †P<0.001 vs MB_{V} in other animal groups.

Figure 7. Representative images of VCAM-1 staining by immunohistochemistry of the thoracic aorta. A, Wild-type mouse on chow diet demonstrating minimal endothelial VCAM-1 staining. B, Wild-type mouse on HCD demonstrating VCAM-1 expression localized to the luminal endothelial surface. C and D, ApoE^{-/-} mouse on chow diet demonstrating VCAM-1 staining particularly on the endothelial surface overlying regions of neointimal thickening. E and F, ApoE^{-/-} mouse on HCD demonstrating robust VCAM-1 staining throughout the aorta but especially on the endothelial surface overlying severe plaque formation and on cells within the neointima.
and magnetic resonance probes.\textsuperscript{21,22} In these studies, VCAM-1 signal in advanced stages of disease decreased with statin therapy, suggesting that the effects of therapy could be monitored with molecular imaging.\textsuperscript{22} Information from microbubble targeting is different from these diffusible tracers in that only endothelial VCAM-1 expression will be detected.

For targeting purposes, monoclonal antibodies against the extracellular domain of VCAM-1 were conjugated to the surface of the microbubbles. This construct is characterized by an average of \(>50\,000\) antibodies per microbubble and a surface density of several thousand per \(1\,\mu\text{m}^2\). Although an antibody-based strategy for targeting has several advantages, including excellent bond dissociation constants, it also has potential problems. One major concern is the relatively low bond formation rates for antibodies that could potentially preclude adequate attachment of rapidly transiting particles in high-shear vessels where contact time is short. Most of the previous studies using targeted microbubbles for molecular imaging have concentrated on molecular targets present in venules of tissues where wall shear stresses and flow velocities are relatively low. In the mouse aorta, peak wall shear stress can reach up to 80 to 90 dynes/cm\(^2\),\textsuperscript{23,24} and the pulsatile variations in flow and thus wall shear stress are high. Despite this problem, there has been successful targeting of smaller echogenic liposomes to vascular surface epitopes in large animal models of atherosclerosis.\textsuperscript{25,26} These studies demonstrated conclusively that endothelial cell adhesion molecules could be targeted with acoustically active compounds, albeit with high-dose upstream administration.

In the present study, flow-chamber experiments demonstrated that VCAM-1–targeted microbubble attachment efficiency was very low during continuous high shear. However, a marked increase in attachment occurred when very high shear was interrupted briefly. Resumption of flow at high shear stress did not dislodge these microbubbles even at the maximum shear rate (12 dynes/cm\(^2\)) that could be withstood without detachment of the SVECs from fibronectin-coated plates. Flow-chamber experiments with precipitated Fc-VCAM-1 chimera have demonstrated the ability of VCAM-1–targeted microbubbles to firmly adhere even at shear rates of 50 and 90 dynes/cm\(^2\) (unpublished data). However, our en face microscopy studies of the aortic arch 10 minutes after intravenous injection of fluorescent microbubbles provided the best evidence that microbubbles could attach in high density to the aortic arch in vivo despite high peak shear stresses during systole.

**Evaluation of Disease Severity**

Molecular imaging of VCAM-1 has the potential to diagnose inflammatory processes that initiate atherosclerosis long before symptoms arise. Although temporal characterization was not performed in this study, our data showing VCAM-1–targeted microbubble attachment and signal enhancement in wild-type mice on HCD without evidence of plaque development suggest that early inflammatory changes can be detected. The finding that targeted microbubble attachment and signal enhancement was much greater in ApoE\textsuperscript{−/−} mice on HCD indicates that various degrees of inflammatory response can be discerned. These mice had not only the greatest extent of endothelial VCAM-1 expression but also the most severe form of disease in terms of plaque burden and the number of VCAM-1–expressing cells (macrophages) within the plaque. In these mice, both CEU and en face microscopy were consistent with a diffuse and widespread attachment of VCAM-1–targeted microbubbles, the density of which was within the dynamic range for detection of microbubbles attached to a 2-dimensional surface.\textsuperscript{27} The diffuse nature of attachment suggests that a surrogate large vessel may be used for evaluation when vascular inflammatory status is severe, although this was not directly tested. In ApoE\textsuperscript{−/−} mice on chow diet, attachment of microbubbles targeted to VCAM-1 was more pronounced in regions of atherosclerotic plaque, consistent with reports on upregulation of VCAM-1 predominantly in regions prone to plaque development.\textsuperscript{11} In the control wild-type mice on Chow diet, attachment of VCAM-1–targeted microbubbles was not different from control microbubbles, reflecting low or absent expression of VCAM-1.\textsuperscript{1,12} This latter finding is important when considering the need for disease specificity (low false-positive rate) required for a screening test.

**Study Limitations**

Although the spatial resolution of the CEU methods of this study was adequate to localize signal enhancement to the aorta, it was not sufficient to determine whether VCAM-1–targeted microbubbles colocalized with plaque formation in the ApoE\textsuperscript{−/−} models. Likewise, on CEU imaging, we could not determine how much of the signal originated from vasa vasorum or plaque neovascular endothelium. However, the ApoE\textsuperscript{−/−} model does not necessarily recapitulate the degree of vasa vaso proliferation in humans, and most of the adhesion on en face microscopy appeared to be on the luminal surface. In a related matter, the acoustic pressure profile of the beam elevational plane indicated that microbubbles attached anywhere in the circumference of the aortic wall would be exposed to a minimum pressure of \(\approx96\,\text{kPa}\) and should be detectable within the beam profile. Hence, microbubbles retained in areas other than the greater or lesser curvature appeared stationary in the center of the lumen. All of these limitations are related to scale, and it is anticipated that imaging in larger animal models or in humans will provide such spatial information.

To the best of our knowledge, this study is the first to use low-power continuous imaging for in vivo targeted ultrasound. Greater signal enhancement could be expected for high-power imaging. When microbubbles are ligated to cells, there can be physical impairment of microbubble oscillation in the acoustic field and subsequent dampening of their signal that appears to be less for high versus low power imaging.\textsuperscript{27} However, the potential for motion artifact in the mouse aorta from respiration on the initial
imaging frame precluded the use of high-power imaging techniques. In terms of shear forces, imaging was performed in anesthetized mice, and shear stress may have been lower than in conscious animals because of the effects of anesthesia. On the basis of our Doppler and B-mode imaging data, we do not believe that there were any significant differences in shear between the animal groups. It is important to realize that one would expect an even greater attachment efficiency in humans. The large-vessel shear stresses in humans are more than an order of magnitude lower than in mice,24 and low-shear vasa vasor proprietary study using intravascular ultrasound. J Am Coll Cardiol. 2006;47:672–677.


CLINICAL PERSPECTIVE

Atherosclerosis often progresses silently for decades before becoming clinically evident. Accordingly, there is growing interest in the ability to detect the vascular inflammatory responses that play a critical role in plaque development and instability. Specifically, methods are being developed to image molecular processes that participate in the pathogenesis of coronary and peripheral artery disease to detect disease early, to improve risk stratification, and to evaluate responses to novel inflammation-targeted therapies. With contrast-enhanced ultrasound, our targeted probes are not able to reach components within the plaque matrix. Hence, to evaluate disease activity in this study, we targeted microbubbles to vascular cell adhesion molecule-1, an endothelial cell adhesion molecule involved in leukocyte trafficking to atherosclerotic lesions. We studied 4 different mouse groups with different degrees of atherosclerotic lesion severity and vascular cell adhesion molecule-1 expression. Microbubble attachment and signal enhancement were different between these groups and varied according to disease severity. These findings indicate that contrast ultrasound molecular imaging of vascular cell adhesion molecule-1 is capable of quantifying vascular inflammatory changes that occur in different stages of atherosclerosis. This method may be potentially useful for early risk stratification according to inflammatory phenotype in patients because it is both quantitative and specific for disease activity. Practical advantages of this approach are that it is rapid, taking only minutes to perform, and relatively inexpensive.
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