Molecular Imaging of Atherosclerotic Plaques Targeted to Oxidized LDL Receptor LOX-1 by SPECT/CT and Magnetic Resonance

Dayuan Li, MD, PhD; Amit R. Patel, MD; Alexander L. Klibanov, PhD; Christopher M. Kramer, MD; Mirta Ruiz, MD; Bum-Yong Kang, PhD; Jawahar L. Mehta, MD, PhD; George A. Beller, MD; David K. Glover, PhD*; Craig H. Meyer, PhD*

**Background**—The oxidized low-density lipoprotein receptor (LDLR) LOX-1 plays a crucial role in atherosclerosis. We sought to detect and assess atherosclerotic plaque in vivo by using single-photon emission computed tomography/computed tomography and magnetic resonance imaging and a molecular probe targeted at LOX-1.

**Methods and Results**—Apolipoprotein E−/− mice fed a Western diet and LDLR−/− and LDLR−/−/LOX-1−/− mice fed an atherogenic diet were used. Imaging probes consisted of liposomes decorated with anti–LOX-1 antibodies or nonspecific immunoglobulin G, 111indium or gadolinium, and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine fluorescence markers. In vivo imaging was performed 24 hours after intravenous injection (150 μL) of LOX-1 or nonspecific immunoglobulin G probes labeled with either 111indium (600 μCi) or gadolinium (0.075 mmol/kg), followed by aortic excision for phosphor imaging and Sudan IV staining, or fluorescence imaging and hematoxylin/eosin staining. The LOX-1 probe also colocalized with specific cell types, apoptosis, and matrix metalloproteinase-9 expression in frozen aortic sections. Single-photon emission computed tomography/computed tomography imaging of the LOX-1 probe showed aortic arch “hot spots” in apolipoprotein E−/− mice (n = 8), confirmed by phosphor imaging. Magnetic resonance imaging showed significant Gd enhancement in atherosclerotic plaques in LDLR−/− mice with the LOX-1 (n = 7) but not with the nonspecific immunoglobulin G (n = 5) probe. No signal enhancement was observed in LDLR−/−/LOX-1−/− mice injected with the LOX-1 probe (n = 5). These results were confirmed by ex vivo fluorescence imaging. The LOX-1 probe bound preferentially to the plaque shoulder, a region with vulnerable plaque features, including extensive LOX-1 expression, macrophage accumulation, apoptosis, and matrix metalloproteinase-9 expression.

**Conclusions**—LOX-1 can be used as a target for molecular imaging of atherosclerotic plaque in vivo. Furthermore, the LOX-1 imaging signal is associated with markers of rupture-prone atherosclerotic plaque. (Circ Cardiovasc Imaging. 2010;3:464-472.)

**Key Words:** molecular imaging ■ LOX-1 ■ atherosclerotic plaque ■ SPECT ■ MRI

Atherosclerosis is a major cause of many cardiovascular disease states, including myocardial ischemia, acute myocardial infarction, and stroke. Many studies have demonstrated that oxidized low-density lipoprotein (ox-LDL) plays a critical role in atherosclerosis. The ox-LDL receptor (LDLR) LOX-1 mediates the pathologic effects of ox-LDL in atherosclerotic lesions. LOX-1 is a type II transmembrane protein with a short, intracellular cytoplasmic tail and a long, extracellular domain containing a C-type lectin-like structure. Binding of ox-LDL to LOX-1 induces apoptosis and expression of adhesion molecules; activates the inflammatory cascade; and causes expression of matrix metalloproteinases (MMPs). These pathologic effects of LOX-1 not only initiate atherosclerotic lesion formation but also contribute to the vulnerability of a plaque to rupture. A recent study demonstrated that LOX-1 deficiency significantly decreases the formation of atherosclerotic lesions.

**Clinical Perspective on p 472**

The key pathologic steps involved in the process of a stable atherosclerotic plaque changing into a ruptured plaque remain poorly understood. Vulnerable plaques are generally nonobstructive, asymptomatic lesions that may abruptly rupture and induce thrombotic occlusion, leading to tissue ischemia and...
its attendant sequelae. Plaque vulnerability is characterized by a thin fibrous cap, accumulation of inflammatory cells, apoptosis, intraplaque hemorrhage, and neovascularization. There are currently no established noninvasive methods for identifying the rupture-prone atherosclerotic plaque in the living animal. The rapidly evolving field of molecular imaging promises important advances in the diagnosis, characterization, and pharmacological treatment of vascular disease. Magnetic resonance imaging (MRI) is a modality that is well suited to vascular imaging, as it can provide anatomic, structural, and functional data on the arterial wall. Other molecular imaging techniques, such as positron emission tomography and single-photon emission computed tomography (SPECT/CT), also seem to be able to accurately visualize and even quantify features of plaque vulnerability and its pathophysiologic processes. Several molecular imaging targets, including apoptosis, macrophages, ox-LDL, high-density lipoprotein, matrix metalloproteinases (MMPs), and αvβ3 integrin, have shown the potential to detect atherosclerotic plaque at risk in vivo.

On the basis of the critical role of LOX-1 and its high expression in atherosclerotic plaque, we have targeted LOX-1 to detect atherosclerotic plaques in vivo. We tested LOX-1 imaging probes by using the same carrier liposomes for both micro-SPECT/CT and MRI studies. We further studied the colocalization of the LOX-1 imaging signal with vulnerable plaque markers of apoptosis, macrophage density, and MMP-9 expression in atherosclerotic plaque.

### Methods

#### Imaging Probes

Liposomes consisted of 1,2-dioleoyl-sn-glycero-3-phosphocholine (Avanti Lipids, Alabaster, Ala), cholesterol (Sigma, St. Louis, Mo), N-hydroxysuccinimide ester of carboxypolyethylene glycol 3400–di-tetraroylphosphatidylethanolamine (Shearwater Polymers, Huntsville, Ala), and 1,1'di-octadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI, Invitrogen) at a mass ratio of 50:25:25:1. Liposomes made as described earlier were coupled with an anti-LOX-1 or nonspecific immunoglobulin G (nIgG) antibody (R&D Systems), 250 μg in 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 8.0, overnight at 4°C so that covalent coupling between the protein amino group and activated carbonyl group of the polyethylene glycol–lipid would occur. The compounds were centrifuged at 45 000 rpm (Beckman) for 30 minutes to remove free antibodies. There were on average 80 antibodies coupled to each liposome. For the MRI study, diethylamidinepentaacetic acid αω-bis(8-stearylamido-3,6) gadolinium complex (Sigma) was added at a 1:1 mass ratio with phosphatidylycholine. The average amount of Gd atoms per liposome was ~150 000. For indium 111 labeling, liposomes carried a trace amount of diethylamidinepentaacetic acid–phosphatidylethanolamine. The liposome preparations were mixed with 111In (Perkin-Elmer LS) in acetate buffer (0.5 mmol/L, pH 6.0) for 2 hours at room temperature and then filtered by a single passage through a Sephadex G50 gel column under centrifugation at 1000g for 1 minute. Finally, the immunoliposome pellets (probes) were dispersed in phosphate-buffered saline (PBS) buffer. The imaging probe consisted of liposomes decorated with anti-LOX-1 antibody (LOX-1 probe) or nonspecific nIgG (nIgG probe). In (SPECT) or Gd (MRI), and DiI fluorescence markers. The particle size was not significantly different before and after protein coupling (144±6 vs 140±71 nm).

#### Dot Blot Analysis

LOX-1 antigen (200 ng, R&D Systems) was placed on a nitrocellulose membrane, dried, and blocked with 3% bovine serum albumin (BSA) for 1 hour. The membranes were incubated with serial dilutions (6.25 to 400 nmol/L of imaging probes) of 111In-liposome–LOX-1 antibody-DiI at room temperature for 2 hours and then exposed to a phosphor imaging plate for 90 minutes, followed by gamma well counting of each dot.

#### Solid-Phase Binding Assay

LOX-1 antigen (200 ng in 0.1 mL PBS) was placed into each well of a standard 96-well ELISA plate overnight, washed with PBS, and then blocked with 3% BSA for 1 hour. Serial dilutions (0.03 to 1.8 μmol/L of imaging probes) of Gd-liposome–LOX-1 antibody-DiI were added to each well and incubated at room temperature for 2 hours. The plate was washed with PBS 4 times. Fluorescence intensity of antigen-bound probes was determined with a microplate reader (Gemini XS, Molecular Devices). A control probe experiment with Gd-liposome-nIgG-DiI was performed in parallel to evaluate nonspecific binding.

#### Animal Protocol

The University of Virginia animal care and use committee approved all animal experiments. Apolipoprotein (apo) E−/− mice were fed a Western diet (Harlan) for 20 weeks and used for SPECT/CT imaging. Mice were anesthetized with isoflurane and injected intravenously with 150 μL (average, 0.12 mmol/L of probes) of LOX-1 or nIgG probe with an average of 600 μCi of 111In. SPECT/CT imaging was performed 24 hours after injection followed by aortic excision. Aortas (n=6) were either longitudinally opened, exposed to the phosphor imaging plate for 90 minutes, and stained with Sudan IV to identify atherosclerotic lesions or (n=6) fixed with 4% paraformaldehyde–PBS solution overnight and then sent to the University of Virginia Core Pathology Laboratory for frozen sectioning.

LDLR−/− (Jackson Laboratories, Bar Harbor, Me) and LDLR+/−/LOX-1−/− mice were fed an atherogenic diet (Harlan) for 16 weeks. Mice were anesthetized with isoflurane and intravenously injected with 150 μL (average, 0.26 mmol/L of probes) of LOX-1 (n=12) or nIgG probe (n=5) with 0.075 mmol Gd per kilogram. MRI (7.0 T, Clinscan, Bruker/Siemens) was performed at baseline and again 24 hours after injection. The aortas were then excised for frozen sectioning to examine binding of the probe in the atherosclerotic plaques and for hematoxylin/eosin (H&E) staining and immunostaining.

#### Blood Pool Clearance and Tissue Biodistribution of Probes

Wild-type C57BL/6 9 (n=6) and apo E−/− (n=8) mice were anesthetized with isoflurane and intravenously injected with the LOX-1 or nIgG probe with ~600 μCi of 111In. Five microliters of blood was collected at 15 and 30 seconds; 1, 15, and 30 minutes; and 1, 2, 4, and 24 hours from the tail vein. Blood pool activity was corrected for decay and normalized by dividing by the initial blood pool counts. The normalized blood pool data were analyzed by biexponential (2-compartment) curve fitting. Twenty-four hours after injection, tissues from the heart, lung, liver, fat, muscle, kidney, spleen and gut and from urine were collected. Decay-corrected tissue radioactivity was calculated as gamma counts per milligram tissue wet weight per time unit and then divided by blood pool radioactivity at 24 hours.

#### Confocal Microscopy

Frozen aortic sections were washed with PBS buffer to remove dextrone and then blocked with 3% BSA for 1 hour. The sections were then incubated with a fluorescein isothiocyanate–absorbed rabbit anti-mouse macrophage antibody (1:50 dilution, Cederlane Laboratories) and a Dylight 633 Red– (Thermo Scientific) labeled goat anti-rabbit antibody to smooth muscle cells (1:100 dilution, Santa Cruz) diluted in 5% BSA overnight at 4°C. The sections were then washed in PBS buffer 4 times for 5 minutes each and incubated with 4',6-diamidino-2-phenylindole (1 μg/mL) for 10 minutes. The sections were washed again with PBS and mounted with an antifade medium. Confocal imaging was performed within 24 hours with a
Zeiss LSM 510 META microscope in an inverted configuration. The detectors were configured for blue, green, red, and far-red emission detection (bandpass filters 420 to 480 nm, 505 to 530 nm, 560 to 615 nm, and 650 nm). Pinhole settings were adjusted for equal “optical sections.”

**Fluorescent Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick End-Labeling Staining**

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling and propidium iodide staining were performed as described previously.10,24 In brief, frozen sections on slides were incubated with 0.3 U/µL terminal deoxynucleotidyl transferase and 0.04 nmol/µL fluorescein-12-dUTP (Promega) in terminal deoxynucleotidyl transferase buffer for 60 minutes at 37°C. Unincorporated fluorescein-dUTP was removed, and the slides were immersed in 1 ferase buffer for 60 minutes at 37°C. Unincorporated fluorescein-fluorescein-12-dUTP (Promega) in terminal deoxynucleotidyl transferase buffer for 60 minutes at 37°C. Unincorporated fluorescein-dUTP was removed, and the slides were immersed in 1 µg/mL propidium iodide in PBS for 15 minutes. The negative controls were performed without terminal deoxynucleotidyl transferase enzyme.

**Fluorescent Immunostaining**

Details of immunostaining have been provided previously.10,24 In brief, 5-µm-thick frozen sections from aortas were incubated with rabbit anti-mouse MMP-9 antibody (sc-6840, Santa Cruz) overnight at 4°C. Sections were rinsed in PBS buffer 4 times and incubated with secondary antibody goat anti-rabbit IgG–fluorescein isothiocyanate (Santa Cruz). Sections were rinsed with PBS buffer 4 times and incubated with secondary antibody goat anti-rabbit IgG–fluorescein isothiocyanate (Santa Cruz). Sections were rinsed with PBS buffer 4 times and incubated with secondary antibody goat anti-rabbit IgG–fluorescein isothiocyanate (Santa Cruz). Sections were rinsed with PBS buffer 4 times and incubated with secondary antibody goat anti-rabbit IgG–fluorescein isothiocyanate (Santa Cruz).

**SPECT/CT Imaging Protocol**

SPECT/CT imaging was performed with a dual-head camera with 1-mm pinhole apertures. A complete SPECT data set consisted of 60 projections at 6° increments at 30 seconds per projection. The images were reconstructed according to a maximum-likelihood expectation maximization algorithm. CT was performed before (without contrast) and after (with contrast) SPECT imaging. A complete CT data set consisted of 216 projection images acquired at 1° increments for 5 minutes. The intravascular agent Fenestra (catalog No. LC-131, ART Inc) was used to provide CT contrast.

**MRI Scan Protocol**

MRI of the ascending aorta was performed with a T1-weighted black-blood spiral gradient-echo sequence (1.2-ms echo time, 90° flip angle, 3×3-cm field of view, 135 interleaves, 4.1-ms readout window, 67-µm spatial resolution) with 11 contiguous 0.5-mm-thick slices. Cardiac-gated double-inversion pulses for suppression of the blood pool were interleaved with cardiac-gated spiral readouts, resulting in an effective repetition time for the readouts of 2 R-R intervals. Four signal averages with cardiac and respiratory gating were used, for a total imaging time of 2.5 minutes per slice. For the postinjection scan, the slices were matched to the baseline preinjection scan with the origin of the left main and left anterior descending coronary arteries as anatomic landmarks. To quantify the MRI results, signal intensity was measured in 4 aortic wall regions of interest as well as the aortic lumen and muscle on each slice at both time points. The regions of interest were manually selected in 4 quadrants around the aorta and matched at the 2 time points.19,20 The standard deviation of noise was also recorded for each slice. These measurements were recorded for all slices at every time point imaged. The contrast-to-noise ratio (CNR) of the aortic wall to lumen was calculated for each slice: percent CNR=(postinjection CNR−preinjection CNR)/preinjection CNR. The normalized enhancement ratio (NER) was defined as the average postcontrast signal intensity from 4 regions of interest within the aortic wall divided by the muscle signal intensity in the same slice and then divided by the precontrast signal intensity: percent NER=(NER−1)×100. In this study, percent CNR and percent NER were used to present the summarized MRI data.25

**Data Analysis**

Data represent the mean of at least 3 independently performed experiments. Data are presented as mean±SD. Data were analyzed by ANOVA, followed by a Bonferroni correction. A probability value of <0.05 was considered statistically significant.

**Results**

**Binding Analysis of Probes In Vitro**

As shown in Figure 1, anti–LOX-1 antibody–linked liposomes successfully bound to LOX-1 antigen-coated surfaces. This was

---

**Table. Mathematical Modeling Parameters (Mean±SEM)**

<table>
<thead>
<tr>
<th>Mouse Strain and Probes</th>
<th>C0</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>R²</th>
<th>θ₁α, h</th>
<th>θ₁β, h</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lox-1 probe</td>
<td>63.3±6.8</td>
<td>9.55±0.94</td>
<td>40.8±5.4</td>
<td>0.64±0.25</td>
<td>0.92±0.07</td>
<td>0.44±0.17</td>
<td>6.62±0.65</td>
</tr>
<tr>
<td>nlgG probe</td>
<td>61.9±12.3</td>
<td>39.9±15.4</td>
<td>41.1±12.6</td>
<td>0.66±0.11</td>
<td>0.99±0.01</td>
<td>1.2±0.95</td>
<td>3.91±1.75</td>
</tr>
<tr>
<td>Apo E °*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lox-1 probe</td>
<td>48.6±7.7</td>
<td>19.3±6.78</td>
<td>54.2±8.2</td>
<td>1.02±0.53</td>
<td>0.98±0.0</td>
<td>0.41±0.07</td>
<td>22.9±4.7</td>
</tr>
<tr>
<td>nlgG probe</td>
<td>46.2±3.9</td>
<td>16.9±6.45</td>
<td>60.0±3.9</td>
<td>0.16±0.01</td>
<td>0.99±0.01</td>
<td>0.11±0.01°</td>
<td>11.8±4.5</td>
</tr>
</tbody>
</table>

C0, C1, C2, and C3 are the parameters obtained from biexponential curve fitting of the blood clearance data with an equation of the form y=C0×exp(−x/C1)+C2×exp(−x/C3). R² is the coefficient of determination; θ₁α and θ₁β are the respective α and β elimination-phase time constants.

*P=0.01 vs LOX-1.
demonstrated by dot blot analysis with the LOX-1 probe (\(^{111}\text{In}-\text{liposome–LOX-1 antibody-DiI}\)) (left). The fluorescence-labeled solid-phase binding assay also showed that the LOX-1 probe (Gd-liposome–LOX-1 antibody-DiI) bound to LOX-1 antigen in a sigmoid curve pattern, indicative of specific binding, whereas the nIgG probe (Gd-liposome-nIgG-DiI) showed very little binding to LOX-1 antigen (right).

**Blood Pool Clearance and Tissue Biodistribution In Vivo**

The parameters obtained from biexponential curve fitting of the blood clearance data are presented in the Table, along with the calculated \(\alpha\) and \(\beta\) elimination half-lives. As shown, the LOX-1 and nIgG probes had similar blood clearance rates in wild-type C57BL/6 mice (Figure 2, upper left). However, in the apo E\(^{-/-}\) mice, the LOX-1 probes cleared more slowly than did the nIgG probes initially after injection (\(P<0.01\)). There was no significant difference in blood pool activity of the 2 probes at 24 hours after injection (Figure 2, upper right). Based on the blood pool clearance data, SPECT/CT and MRI were performed at 24 hours after injection of the probe. As expected, both probes largely accumulated in the liver and spleen because of their liposome nature and the presence of Fc fragments on the antibody or IgG molecules on the liposome surface.

**LOX-1 Expression and LOX-1 Probe Binding in Plaque In Vivo**

By immunostaining, we showed that LOX-1 was highly expressed in atherosclerotic plaques, especially in the shoulder area (Figure 3, left). Consistent with this finding, most of the LOX-1 probe binding was also observed in this same region (Figure 3, middle). Notably, we found that the LOX-1 probe did not bind to every atherosclerotic plaque in vivo. In contrast to the extensive binding of the LOX-1 probe, there was negligible nIgG probe binding to the atherosclerotic plaque in vivo (Figure 3, right).

**Colocalization of LOX-1 Probe Binding to Cells Within Atherosclerotic Plaque In Vivo**

To further characterize specific cell lines within the plaque to which the LOX-1 probe binds, we performed fluorescent immunostaining for macrophages and smooth muscle cells in frozen aortic sections taken from apo E\(^{-/-}\) mice injected with LOX-1 probes. Confocal microscopy showed that a vast amount of LOX-1 probe colocalized with macrophages in the atherosclerotic plaque, with little colocalization with proliferated smooth muscle cells (Figure 4).

**LOX-1 Probe Binding, Apoptosis, and MMP-9**

Next, we examined the relation between the in vivo LOX-1 imaging signal and other typical markers of a vulnerable plaque, including apoptosis and MMP-9 expression. As shown in Figure 5, LOX-1 probe binding was colocalized with apoptotic cells (Figure 5, left) as well as with MMP-9 (right). Notably, there were few apoptotic cells or little MMP-9 expression in regions of the plaque with minimal or no LOX-1 probe binding.

**SPECT/CT Imaging, Phosphor Imaging, and Sudan IV Staining**

As shown in Figure 6, there were no “hot spots” detected by in vivo SPECT or ex vivo phosphor imaging in apo E\(^{-/-}\) mice injected with the nIgG probe (top row). In contrast, all mice injected with the LOX-1 probe had hot spots in the atherosclerotic region of the aortic arch by in vivo SPECT...
and ex vivo phosphor imaging (bottom row). There were no differences in the extent of atherosclerotic lesions between the 2 groups as determined by Sudan IV staining.

**MRI, Fluorescence Imaging, and H&E Staining**

Strong postinjection Gd enhancement was visualized in the aortic root and arch in LDLR<sup>−/−</sup> mice injected with the LOX-1 probe (Figure 7, left). These findings were confirmed by ex vivo fluorescence imaging of frozen sections that showed LOX-1 probe binding in the same area. The size of atherosclerotic plaque was confirmed by H&E staining. Post-Gd enhancement was not seen in the LDLR<sup>−/−</sup> mice injected with the nIgG probe (Figure 7, middle). Furthermore, the specificity of LOX-1 probe binding was confirmed in LDLR<sup>−/−</sup>/LOX-1<sup>−/−</sup> double-knockout mice that showed no post-Gd enhancement and no LOX-1 probe binding to plaque (Figure 7, right). These mice also showed fewer atherosclerotic lesions on H&E staining, in agreement with previous studies.<sup>10</sup> Quantitative MRI data (right) showed that LDLR<sup>−/−</sup> mice injected with the LOX-1 probe had a significantly higher CNR and NER than did the other 2 control groups, LDLR<sup>−/−</sup>/LOX-1<sup>−/−</sup> mice injected with the nIgG probe and LDLR<sup>−/−</sup>/LOX-1<sup>−/−</sup> mice injected with the LOX-1 probe.

**Discussion**

Specific noninvasive techniques are needed to detect atherosclerotic plaque vulnerability and to monitor changes in these parameters. Development of such approaches requires delineation of appropriate markers of atherosclerosis and use of noninvasive imaging tags directed at these markers. To date, most invasive (intravascular ultrasound) and noninvasive (CT angiography) techniques for plaque imaging identify only the presence and extent of atherosclerotic plaque and do not image specific targets related to vulnerability.

Several studies have suggested that activation of the ox-LDLR LOX-1 induces endothelial dysfunction, enhances ox-LDL uptake in monocytes/macrophages, and induces a state of oxidative stress. Accordingly, it has been suggested that LOX-1 is involved in the initiation and progression of atherosclerotic plaque.<sup>3–10,24</sup> Indeed, recent studies show that deletion of LOX-1 reduces the progression of atherosclerosis.<sup>10</sup> Furthermore, LOX-1 activates MMPs,<sup>9</sup> resulting in collagen degradation and initiation of plaque rupture, the most proximate cause of acute coronary syndromes. Circulating levels of soluble LOX-1 are increased in patients with unstable coronary syndromes.<sup>26</sup>

We validated the molecular imaging of LOX-1 protein to detect atherosclerotic plaque by SPECT/CT and MRI techniques in 2 different models of atherosclerosis, the apo E<sup>−/−</sup> and LDLR<sup>−/−</sup> mouse strains fed a high-cholesterol diet. We found that the LOX-1 probe, given intravenously, specifically bound to atherosclerotic plaques, especially in the cap and shoulder areas, and could be clearly detected by both SPECT/CT and MRI of the aortic arch. Importantly, we found that the LOX-1 signal in the atherosclerotic plaque colocalized with macrophages, apoptotic cells, and MMP-9 expression. It is well known that plaques that are prone to rupture contain large numbers of inflammatory and apoptotic cells, and these regions secrete large amount of MMP-9.

**Molecular Imaging Probes for Detecting Atherosclerotic Plaque at Risk**

The ideal probe should target proteins expressed in the atherosclerotic plaque specifically and extensively, and it should have a high binding affinity, high sensitivity, and high selectivity to target(s) in the atherosclerotic plaque. On the basis of these criteria, various investigators have targeted different proteins and components of plaque to image athero-
sclerotic plaque at risk in vivo. Apoptosis, an important component of atherosclerosis, results in a loss of cell density in the plaque, which may contribute to plaque instability. Sarai et al. used radiolabeled annexin V to detect apoptosis in the atherosclerotic plaque in a rabbit model. It is now well recognized that atherosclerotic lesions contain a large number of monocytes/macrophages as part of the inflammatory process, and the number of inflammatory cells predicts vulnerability of the plaque to rupture. Accordingly, some studies targeted macrophages with Gd immune micelles for MRI in apo E mice and iodinated nanoparticles dispersed with surfactant for CT imaging in rabbits. As mentioned earlier, enhanced MMP expression and activity cause degradation of collagen in the cap and thus increase the tendency of the plaque to rupture. Accordingly, some studies have targeted MMPs to detect atherosclerotic plaque in apo E and LDLR mice. Other studies have targeted ox-LDL, vascular cell adhesion molecule-1, fibrin-binding peptide derivatives, and LOX-1 antibody to detect atherosclerotic plaque in a rabbit model. However, use of this imaging probe was limited by low spatial resolution and a poor signal-to-noise ratio because the half-life of 99mTc is 6 hours and an antibody usually takes >6 hours to bind to an antigen in vivo.

In the current study, we constructed imaging probes by using liposome vectors to carry the LOX-1 antibody, the fluorescent marker DiI, and 111In or Gd. The size of the liposome was kept at 200 nm so that the liposomes could leave the vasculature, enter the interstitial space, and bind to the appropriate components of the atherosclerotic plaque. The liposome–LOX-1 antibody highly and specifically bound to the LOX-1 antigen, whereas very little liposome-nIgG bound. The affinity of the LOX-1 antibody was unaffected by the coupling process. The half-life of 111In is 2.8 days. The majority of the LOX-1 probe cleared from the blood pool 24 hours after injection.
hours after injection, providing the rationale for imaging at 24 hours after injection. As expected with liposomes, a significant amount of the probe accumulated in the liver and spleen; however, this did not affect image quality because we scanned from the aortic root to arch.

**LOX-1 Imaging Signal and Vulnerability of Atherosclerotic Plaque to Rupture**

During the last decade, it has become evident that rupture-prone atherosclerotic plaques are seen in a majority of patients with life-threatening coronary syndromes. It is widely appreciated that the shoulder region of plaque is a common site in plaque rupture because it has more inflammation, apoptosis, shear stress, and gene expression.43–45

We found that the LOX-1 probe was localized primarily in the shoulder region of plaques in atherosclerotic LDLR−/− mice. This finding was correlated with the distribution of LOX-1 expression seen by immunostaining. Importantly, we found that the LOX-1 probe colocalized with macrophages, apoptotic cells, and MMP-9—expressing cells. A recent study in atherosclerotic rabbits found that in mice injected with the LOX-1 antibody accumulated selectively in atheromatous lesions with large numbers of macrophages and extracellular lipid deposits but fewer smooth muscle cells and collagen fibers. Our results are important because we showed colocalization of the LOX-1 probe to rupture-prone atherosclerotic regions in murine models that are often used for developing molecule-targeted diagnostic and therapeutic approaches.

**Multimolecular Imaging Targeted to LOX-1 for Characterizing Atherosclerotic Plaque**

No single imaging modality can provide overall structural, functional, and molecular information, as each modality has its own strengths and weaknesses. Nuclear imaging has high sensitivity but low spatial resolution. MRI has high spatial resolution, providing clear information on anatomy and function, but the sensitivity is not as high as with nuclear imaging. Multimodality molecular imaging techniques can provide complementary information to define the characteristics of atherosclerotic plaque.13,46,47 In the current study, we found that SPECT/CT clearly showed hot spots in the atherosclerotic region of the aortic arch in apo E−/− mice injected with the LOX-1, but not the nIgG, probe. Likewise, MRI demonstrated strong post-Gd enhancement in atherosclerotic plaques from the aortic root to the arch in LDLR−/− mice injected with the LOX-1 probe with no post-Gd enhancement in the same mice injected with the nIgG probe. Specificity of the LOX-1 imaging signal was further delineated in LDLR−/− mice with LOX-1 abrogation that showed a marked reduction in LOX-1 probe binding and post-Gd enhancement. Previous studies have shown a marked reduction in atherogenesis in these double-knockout mice.10

These data from 2 different mouse models and 2 different imaging modalities adequately validate that LOX-1 can be used as a target for molecular imaging to detect atherosclerotic plaques. Other imaging studies have focused on the abdominal aorta. A weakness of those studies relates to the accumulation of probes in the liver, spleen, and kidney, which are adjacent to the abdominal aorta. To overcome this limitation, we focused on the aortic root and arch. These regions have more extensive atherosclerosis than does the abdominal aorta in mice, but more importantly, one can avoid false-positive data due to probe accumulation in the liver and spleen.

In summary, we show that LOX-1 can be used as a molecular imaging target with high binding affinity, sensitivity, and selectivity in atherosclerotic plaques. LOX-1 targeting provides important information pertaining to the vulnerability of atherosclerotic plaques. These multimodality molecular imaging techniques may evolve into clinically relevant noninvasive tools for detecting and monitoring rupture-prone plaques. These techniques may also be used to assess the efficacy of novel treatments that induce plaque regression and stabilization.

**Acknowledgments**

The authors acknowledge R. Jack Roy and Joseph Pole for assistance with the MRI and SPECT/CT, respectively; Joseph DiMaria for assistance with the animals; and Christopher T. Sica, Weitian Chen, and Hao Tan for assistance with image reconstruction. The authors also acknowledge helpful discussion with Brett Blackman and Kimberly Kelly. Finally, we acknowledge the vision of the late Walter J. Rogers, PhD, who planted the seeds of this work.

**Sources of Funding**

This work was supported by the American Heart Association Grant-in-Aid (2490014), University of Virginia–Coulter Foundation Translational Research Partnership, the Nuclear Cardiology Foundation, and National Institutes of Health grant T32 HL007355. Dr Patel was supported by National Institutes of Health training grant 5T-32 EB003841.

**Disclosures**

Drs Li, Patel, Klibanov, Kramer, Beller, Glover, and Meyer are listed as inventors on US Patent Application Serial No. 61/108,701 titled “Multimodal imaging of atherosclerotic plaque targeted to LOX-1 receptor” filed on October 27, 2008, and US Patent Application Serial No. 61/221,331 filed on June 29, 2009. In addition, Dr Kramer receives research support (>50K) from Siemens Medical Solutions and is a consultant (<50K) for Siemens Medical Solutions. Drs Ruiz, Kang, and Mehta report no conflicts of interest.

**References**

28. cardiac imaging.
CLINICAL PERSPECTIVE

Oxidized low-density lipoprotein plays a critical role in atherosclerosis, and its effects are mediated by activation of its specialized receptor, LOX-1. LOX-1 induces apoptosis, expression of adhesion molecules and matrix metalloproteinases, and in general, activates inflammation. LOX-1 activation not only initiates lesion formation but also contributes to the vulnerability of the plaque to rupture. Thus, a noninvasive molecular imaging approach to identify LOX-1 in the live animal would be advantageous to study the atherosclerotic process early in its natural history to enable optimal therapies to prevent acute coronary syndromes. Imaging probes for both single-photon emission computed tomography/computed tomography and magnetic resonance imaging were constructed that consisted of liposomes decorated with anti–LOX-1 antibodies (LOX-1 probe) and either $^{111}$indium or gadolinium, as well as fluorescent markers. In vivo imaging was performed 24 hours after intravenous injection of 150 μL LOX-1 probe in mouse models of atherosclerosis. Single-proton emission computed tomography/computed tomography imaging showed aortic arch hot spots, and magnetic resonance imaging showed significant contrast enhancement in aortic atherosclerosis. Binding was shown to be specific for LOX-1, and the probe bound preferentially to the plaque shoulder, a region with vulnerable plaque features including extensive LOX-1 expression, macrophage accumulation, apoptosis, and matrix metalloproteinase-9 expression. Thus, this flexible multimodality molecular imaging approach could be used to identify features of vulnerable plaque in vulnerable patients. Moving this approach into the clinic to identify patients at risk for acute coronary syndromes could lead to improved targeting of therapies aimed at reducing inflammation and atherosclerosis to prevent these syndromes.
Molecular Imaging of Atherosclerotic Plaques Targeted to Oxidized LDL Receptor LOX-1 by SPECT/CT and Magnetic Resonance

Dayuan Li, Amit R. Patel, Alexander L. Klibanov, Christopher M. Kramer, Mirta Ruiz, Bum-Yong Kang, Jawahar L. Mehta, George A. Beller, David K. Glover and Craig H. Meyer

_Circ Cardiovasc Imaging_. 2010;3:464-472; originally published online May 4, 2010; doi: 10.1161/CIRCIMAGING.109.896654

_Circulation: Cardiovascular Imaging_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1941-9651. Online ISSN: 1942-0080

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circimaging.ahajournals.org/content/3/4/464

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation: Cardiovascular Imaging_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation: Cardiovascular Imaging_ is online at:
http://circimaging.ahajournals.org/subscriptions/