Molecular Imaging of Angiogenesis in Early-Stage Atherosclerosis With $\alpha_v\beta_3$-Integrin–Targeted Nanoparticles

Patrick M. Winter, PhD; Anne M. Morawski, BS; Shelton D. Caruthers, PhD; Ralph W. Fuhrhop; Huiying Zhang, MD; Todd A. Williams, BS; John S. Allen, BS; Elizabeth K. Lacy, BS; J. David Robertson, PhD; Gregory M. Lanza, MD, PhD; Samuel A. Wickline, MD

Background—Angiogenesis is a critical feature of plaque development in atherosclerosis and might play a key role in both the initiation and later rupture of plaques that lead to myocardial infarction and stroke. The precursory molecular or cellular events that initiate plaque growth and that ultimately contribute to plaque instability, however, cannot be detected with current modalities.

Methods and Results—Atherosclerosis was induced in New Zealand White rabbits fed 1% cholesterol for ~80 days. $\alpha_v\beta_3$-Integrin–targeted, paramagnetic nanoparticles were injected intravenously and provided specific detection of the neovascularization within 2 hours by routine magnetic resonance imaging (MRI) at a clinically relevant field strength (1.5 T). Increased angiogenesis was detected as a 47% enhancement in MRI signal averaged throughout the abdominal aortic wall among rabbits that received $\alpha_v\beta_3$-targeted, paramagnetic nanoparticles. Pretreatment of atherosclerotic rabbits with $\alpha_v\beta_3$-targeted, nonparamagnetic nanoparticles competitively blocked specific contrast enhancement of the $\alpha_v\beta_3$-targeted paramagnetic agent. MRI revealed a pattern of increased $\alpha_v\beta_3$-integrin distribution within the atherosclerotic wall that was spatially heterogeneous along both transverse and longitudinal planes of the abdominal aorta. Histology and immunohistochemistry confirmed marked proliferation of angiogenic vessels within the aortic adventitia, coincident with prominent, neointimal proliferation among cholesterol-fed, atherosclerotic rabbits in comparison with sparse incidence of neovascularization in the control animals.

Conclusions—This molecular imaging approach might provide a method for defining the burden and evolution of atherosclerosis in susceptible individuals as well as responsiveness of individual patients to antiatherosclerotic therapies.

Key Words: magnetic resonance imaging • atherosclerosis • contrast media • angiogenesis

The preponderance of annual mortality in the Western world is caused by atherosclerosis, which presents as cerebrovascular accident or myocardial infarction. In the United States alone, nearly 500,000 cardiac deaths occur each year without warning. Unfortunately, comprehensive evaluation of atherosclerosis generally ensues only after a clinical event that is caused by plaque rupture in the later stages of this disease process. Efforts to prevent the development of clinically significant atherosclerotic disease through dietary, lifestyle, and pharmaceutical means are ongoing, but sensitive and specific metrics of early disease are essentially unavailable.

Angiography, the “gold standard” technique for the detection and characterization of severe atherosclerotic stenosis, has notable limitations inherent to all lumenographic techniques. Other invasive approaches, ie, elastography, thermography, and intravascular ultrasound, are designed to characterize late-stage vascular disease. In contrast, magnetic resonance imaging (MRI) offers a unique, noninvasive opportunity for sensitive detection and characterization of atherosclerotic disease.

The inherently different $T_2$ relaxation times of atheromatous plaque allow MRI to distinguish cholesterol crystals, collagenous cap, and normal media components. High-resolution black-blood techniques provide detailed images of carotid and coronary arterial walls and might anatomically resolve the thinning fibrous caps of vulnerable plaques. Delayed and dynamic contrast-enhanced MRI can provide an index of vascularity in patients with late carotid disease.

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From the Cardiovascular Magnetic Resonance Laboratories (P.M.W., A.M.M., S.D.C., R.W.F., H.Z., T.A.W., J.S.A., E.K.L., G.M.L., S.A.W.), Department of Medicine, Washington University School of Medicine, St Louis, Mo; Philips Medical Systems (S.D.C.), Best, the Netherlands; and the University of Missouri Research Reactor (J.D.R.), Columbia, Mo.

Dr Caruthers is an employee of Philips Medical Systems. Drs Lanza and Wickline are cofounders, board members, and equity holders of Kereos, Inc. R.W. Fuhrhop and J.S. Allen are consultants to Kereos, Inc.

Correspondence to Samuel A. Wickline, MD, or Gregory M. Lanza, MD, PhD, Washington University School of Medicine, Campus Box 8086, 660 S Euclid Ave, St Louis, MO 63110. E-mail saw@howdy.wustl.edu

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These measurements focus on later stages of plaque development that portend or indicate plaque rupture.

In this report, we describe an in vivo, molecular diagnostic imaging approach for quantifying very early plaque development. Angiogenic biomarkers are induced in widespread vascular territories in response to cholesterol feeding, which results in early and critical expansion of the vasa vasorum to support plaque growth. Integrins, such as $\alpha_\beta_3$, are associated with angiogenesis, and atherosclerotic lesions are highly vascular compared with normal vessel tissues. Accordingly, we proposed and developed a paramagnetic nanoparticle contrast agent targeted specifically to $\alpha_\beta_3$-integrins to permit noninvasive molecular imaging of plaque-associated angiogenesis.

**Methods**

**Targeted Nanoparticles**

For in vivo imaging of sparse molecular epitopes of angiogenesis associated with atherosclerosis, we formulated paramagnetic nanoparticles targeted to $\alpha_\beta_3$-integrins. Methods developed in our laboratories were used to prepare perfluorocarbon (perfluorooctylbromide) emulsions encapsulated by a lipid-surfactant monolayer. Approximately 90 000 paramagnetic chelates (gadolinium–dithylenetriamine pentaacetic acid–bis-olate [Gd-DTPA-BOA]) were formulated onto the surface of each particle, as previously described. Paramagnetic nanoparticles were directed to the $\alpha_\beta_3$-integrin with a peptidomimetic vitronectin antagonist (US patent No. 6,130,231). The Arg-Gly-Asp mimetic was coupled at a 1:1 molar ratio to malcimidophenyl-butyramide-polyethylene glycol$\sim$2000 phosphatidylethanolamine resuspended from a dry lipid film in 3 mL of N$_2$-purged, 6 mmol/L EDTA by water-bath sonication for 30 minutes at 37°C to 40°C. This ligand premix was added to the remaining surfactant components, perfluorooctylbromide, and water for emulsification. Nontargeted, paramagnetic nanoparticles were prepared by excluding the targeting ligand, and $\alpha_\beta_3$-targeted, nonparamagnetic nanoparticles were produced by omitting the lipophilic Gd$\text{++}$ chelate. The nominal sizes for each formulation were measured with a submicron particle analyzer (Malvern Zetasizer, Malvern Instruments).

The gadolinium concentrations were quantified by neutron activation analysis conducted at the Research Reactor facility at the University of Missouri. In brief, samples were placed in specialized plastic cuvettes that contained no interfering metals, weighed wet, lyophilized, and reweighed. The mass of gadolinium was determined by measuring the 361-keV $\gamma$-rays from the $\beta$-decay of $^{151}$Gd (t$_{1/2}$=3.66 minutes) produced through neutron capture on $^{150}$Gd. Individual samples and standards were irradiated in a thermal neutron flux of $\sim$5x$10^{11}$ n · cm$^2$ · s$^{-1}$ for 7 seconds, allowed to decay for 30 seconds, and counted on a high-resolution $\gamma$-ray spectrometer for 300 seconds. The minimum detectable amount of gadolinium with this method is reported to be several nanograms.

The actual T$_1$ and T$_2$ relaxivities (r$_1$ and r$_2$, respectively) of the particle formulations were determined with the use of standard inversion recovery and multicue pulse sequences applied to pure samples (nanoparticles present at 59 mmol/L). Samples were placed in a quadrature birdcage coil and imaged with a clinical 1.5-T system (Philips NT Intera CV, Philips Medical Systems).

**Experimental Design**

Male New Zealand White rabbits (Charles River Laboratories, Cambridge, Mass) were fed either 1% cholesterol (n=16) or standard rabbit chow (n=4) for $\sim$80 days. Cholesterol-fed rabbits were divided into 3 groups, each of which received 1 of the following: (1) $\alpha_\beta_3$-targeted, paramagnetic nanoparticles (n=6); (2) nontargeted, paramagnetic nanoparticles (n=4); or (3) pretreatment with $\alpha_\beta_3$-targeted, nonparamagnetic nanoparticles 2 hours before delivery of $\alpha_\beta_3$-targeted, paramagnetic nanoparticles (n=6). The last group was designed to demonstrate the specificity of the $\alpha_\beta_3$-integrin targeting by in vivo competitive blocking of the binding of targeted, paramagnetic nanoparticles. All control diet animals received $\alpha_\beta_3$-targeted, paramagnetic nanoparticles ($\sim$273 nm in diameter [polydispersity=0.15] and contained 6.17 mmol/L gadolinium, or $\sim$94 200 Gd$\text{++}$ atoms per particle. The “ionic-based” r$_1$ and r$_2$ values for paramagnetic nanoparticles expressed per mole Gd$\text{++}$ per liter were 17.7±0.2 and 25.3±0.6 (s · mmol/L)$^{-1}$, respectively, and the “particle-based” relaxivities that reflect the signal effects achievable per individual binding site (ie, particle) were 1 /700 000±100 000 and 2 380 000±120 000 (sec · mmol particle/L)$^{-1}$ for r$_1$ and r$_2$. After baseline MRI, all contrast agents were injected peripherally into the ear vein at a dose of 0.5 mL/kg body weight, and contrast enhancement was followed for 2 hours. Nonspecific “delayed enhancement” between control and fat-fed rabbits was quantified 20 minutes after injection of 0.1 mmol/kg gadolinium-DTPA (Magnevist, Berlex Laboratories, Inc). After imaging, aortas were extracted for histologic assessment, as described later. The experimental protocol was approved by the Animal Studies Committee of the Washington University School of Medicine.

**MRI Protocol**

A 1.5 T clinical magnet (NT Intera CV, Philips Medical Systems) was used, along with a quadrature birdcage radiofrequency receive coil to image the abdominal aorta in vivo before and at 15, 60, and 120 minutes after treatment with paramagnetic nanoparticles. Multislice, T$_1$-weighted, spin-echo, fat-suppressed, black-blood imaging of the aorta was performed from the renal arteries to the diaphragm (repetition time=380 ms, echo time=11 ms, 250×250-μm in-plane resolution, 5-mm slice thickness, number of signals averaged=8). To null the blood signal, “sliding radiofrequency” saturation bands were placed proximal and distal to the region of image acquisition and moved with the selected imaging plane.

**Image Analysis**

We developed an objective and quantitative method for calculating contrast enhancement within the aortic wall for each image slice, based on a customized, semiautomated segmentation program. The aortic lumen was isolated in each 2-dimensional image through the use of a seeded “region-growing” algorithm. The segmented lumen was “dilated” 5 pixels to encompass the entire vessel wall. Further thresholding was used to eliminate background pixels, leaving a binary “mask” of the aortic wall for each slice and time-point combination. Signal intensity was normalized to the signal from a fiducial marker (a gadolinium-DTPA/saline solution in a test tube phantom) that was placed within the field of view. The signal enhancement of the aortic wall and adjacent muscle was calculated slice by slice with respect to the baseline images. General linear modeling with Duncan’s multiple-range testing of group differences (SAS, Inc) was used to determine the significance of differences in MRI signals (P<0.05).

**Histology**

Routine hematoxylin/eosin staining was performed on formalin-fixed, paraffin-embedded sections (4 μm) of aortas. Expression of $\alpha_\beta_3$ integrin in the aortic wall was confirmed by immunohistochemistry of formalin-fixed sections with use of a specific primary antibody (LM609, Chemicon International, Inc) and a secondary antibody developed with an aminoethylcarbazole substrate kit. Platelet and endothelial cell adhesion molecule (PECAM) was stained similarly with CD31 primary antibody (Chemicon International, Inc). Images of neovascularization were digitized under high power (600×) with a Nikon microscope and Nikon DXM1200 camera.

**Results**

**Nanoparticle Characteristics**

Nanoparticles were $\sim$273 nm in diameter (polydispersity=0.15) and contained 6.17 mmol/L gadolinium, or $\sim$94 200 Gd$\text{++}$ atoms per particle. The “ionic-based” r$_1$ and r$_2$ values for paramagnetic nanoparticles expressed per millimole Gd$\text{++}$ per liter were 17.7±0.2 and 25.3±0.6 (s · mmol/L)$^{-1}$, respectively, and the “particle-based” relaxivities that reflect the signal effects achievable per individual binding site (ie, particle) were 1 /700 000±100 000 and 2 380 000±120 000 (sec · mmol particle/L)$^{-1}$ for r$_1$ and r$_2$. After baseline MRI, all contrast agents were injected peripherally into the ear vein at a dose of 0.5 mL/kg body weight, and contrast enhancement was followed for 2 hours. Nonspecific “delayed enhancement” between control and fat-fed rabbits was quantified 20 minutes after injection of 0.1 mmol/kg gadolinium-DTPA (Magnevist, Berlex Laboratories, Inc). After imaging, aortas were extracted for histologic assessment, as described later. The experimental protocol was approved by the Animal Studies Committee of the Washington University School of Medicine.
respectively. Targeted and nontargeted particles exhibited equivalent paramagnetic properties.

**α,β3-Integrin Within Atherosclerotic Aortic Wall**

Figure 1 (top) shows the imaged portion of the aorta in longitudinal profile for a selected animal and transverse slices (bottom) at baseline (Pre) and 120 minutes after (Post) treatment with targeted nanoparticles and the segmented aortic wall mask (Segmented) used to quantify the contrast enhancement (Enhancement) for this slice. The signal in the aortic wall increased after contrast injection (labeled Post and Enhancement), indicating the presence of targeted nanoparticles bound to α,β3-integrin epitopes on the neovasculature. Furthermore, note that the aortic blood pool background was not confounded by signal originating from circulating nanoparticles, which enabled rapid detection of contrast enhancement in the aortic wall. Aortic wall contrast enhancement was variable along the circumference and length of the aorta, as illustrated by the color-coded signal enhancement maps (Figure 2) and longitudinally along the aorta for 3 selected rabbits (Figure 3). However, greater signal enhancement was observed in the cholesterol-fed, targeted rabbits for practically every aortic segment.

Histology of cholesterol-fed rabbit aortas revealed mild intimal thickening after 80 days, which was not appreciated in animals that received the control diet (Figure 4). Estimation of lumen area and wall thickness by MRI was similar for all treatment groups, reflecting the early stage of vascular disease in these animals. Immunohistochemistry revealed expansion of the aortic vasa vasorum (PECAM-positive) among atherosclerotic rabbits in comparison with the controls. Angiogenic vessels, delineated by colocalized staining of α,β3-integrin and PECAM, formed a subpopulation of the total expanded vasculature in the hyperlipidemic aortas, predominately localized to the adventitia-media interface (Figure 4). In control rabbits, a sparse neovasculature was noted in the same regions.

Quantification of the aortic signal enhancement among cholesterol-fed rabbits that received α,β3-targeted, paramagnetic nanoparticles showed a 26±4% and 47±5% increase over baseline at 15 and 120 minutes, respectively, when averaged across the expanse of the aorta for each rabbit (Figure 5, bottom). In cholesterol-fed rabbits that received nontargeted nanoparticles, the aortic wall was enhanced by 19±1% within 15 minutes, but it stabilized from 60 to 120 minutes at 26±1%, which represents about half of the signal augmentation observed for the specific α,β3-targeted enhancement. Competitive blockade of angiogenic α,β3-integrins with targeted, nonparamagnetic nanoparticles reduced the signal enhancement of α,β3-targeted, paramagnetic nanoparticles by at least 50%. In control diet rabbits, aortic wall enhancement from α,β3-targeted nanoparticles paralleled the contrast effect noted among the nontargeted, cholesterol-fed animals. Thus, the overall average signal enhancement in the aortic wall for cholesterol-fed animals was approximately twice that for control diet animals at 120 minutes. The signal enhancement observed in the adjacent skeletal muscle (Figure 5, top) owing to nanoparticles in all treatment groups was negligible relative to that exhibited by the aortic wall.

In contradistinction to the specific MRI enhancement observed with α,β3-targeted nanoparticles, delayed contrast
enhancement of the vessel wall with gadolinium-DTPA revealed no significant difference between cholesterol-fed (91 ± 7%) and control diet (87 ± 3%) animals. Although non-specific contrast agents have been used to distinguish fibrous, necrotic, and calcified tissues associated with complex plaques, they were insensitive to the subtle vascular changes associated with the early development of atherosclerosis.

Discussion
Plaque angiogenesis and expansion of the vasa vasorum are critical processes for the initiation of vascular lesions and the progression of early atherosclerotic inflammatory events driven by cholesterol feeding. These data indicate that targeted, paramagnetic nanoparticles might be used for molecular imaging of inducible $\alpha_\beta_3$-integrins throughout the vascular wall and reaffirm that early atherosclerosis evolves as a diffuse process in response to elevated cholesterol. Targeted characterization of $\alpha_\beta_3$-integrin expression revealed that the early atherosclerotic process manifests considerable heterogeneity within individual aortic segments. Variation in the gross severity of atherosclerosis from the diaphragm to the renal arteries generally corresponded to the calculated MRI signal enhancement and the magnitude of neovascular proliferation observed histologically. These findings are in agreement with an analogous heterogeneous distribution of aortic foam cells in cholesterol-fed rabbits illustrated with “cold-spot” imaging of susceptibility artifacts created by the cellular uptake of nontargeted, iron oxide particles. Although the precise role of $\alpha_\beta_3$-integrin expression in the expansion of the vasa vasorum was not elucidated.

Figure 3. Spatial variation of contrast enhancement after treatment showing longitudinal signal enhancement from renal arteries to diaphragm for 1 representative animal selected from 3 experimental groups: cholesterol (Chol)-fed/targeted, control diet (CNT), and cholesterol-fed/nontargeted.

Figure 4. Immunohistochemistry of $\alpha_\beta_3$-integrin in aorta sections from cholesterol-fed (A, C, D) and control diet (B) rabbits. Thickened intima (I) is observed in cholesterol-fed animal (A, 200×) but not in control animal (B, 200×). Immunostaining of neovascular $\alpha_\beta_3$-integrin (C) and PECAM (D) in aorta from cholesterol-fed animal in A at 600×. Solid arrows delineate $\alpha_\beta_3$-integrin expression, and open arrows mark PECAM expression at interface between media (M) and adventitia (Av). Prevalence of $\alpha_\beta_3$-integrin staining in control rabbit (solid arrows in B) is far less prominent than for cholesterol-fed animal (A).

Figure 5. Quantitative analysis of MRI signal enhancement (percent) from aorta (bottom) and skeletal muscle (top) after treatment with $\alpha_\beta_3$-targeted or nontargeted nanoparticles in cholesterol-fed or control diet groups. *P<0.05 for cholesterol-fed/targeted vs all other groups.
by these data, its detection by noninvasive imaging methods could serve as a useful biochemical signature of the early atherosclerotic condition that might demarcate lesion-prone sites of focal plaque growth.

In the present study, we confirmed the in vivo binding specificity of $\alpha_B\beta_3$-targeted nanoparticles with in vivo competition studies. Pretreatment of cholesterol-fed rabbits with $\alpha_B\beta_3$-targeted, nonparamagnetic nanoparticles blocked the increased signal enhancement from $\alpha_B\beta_3$-targeted, paramagnetic nanoparticles to a level slightly below that obtained for nontargeted, paramagnetic nanoparticles. Passive contrast enhancement among atherosclerotic animals injected with nontargeted nanoparticles might reflect neovascularure-related leakage within the aorta. In rabbits fed a control diet and given $\alpha_B\beta_3$-targeted, paramagnetic nanoparticles, signal enhancement was similar to the nonspecific background level. This low-contrast effect might be partially attributable to a combination of specific targeting and passive leakage associated with sparse neovascular vessels observed histologically (Figure 4).

In the present study conducted at 1.5 T, molecular imaging of angiogenesis in hyperlipidemic rabbits was appreciated within the aortic wall but could not be spatially resolved to a specific vascular layer (eg, adventitia). With the anticipated clinical adoption of 3 T and higher-field MR scanners, greater image resolution is expected, which should improve noninvasive neovascular localization and facilitate clinical risk assessments.

We have previously reported that this nanoparticle emulsion allows sensitive molecular imaging of abundant vascular epitopes (eg, fibrin) with clinical ultrasound and MRI systems.\textsuperscript{13,14,21} In this study, we have shown that paramagnetic nanoparticles can provide sensitive in vivo detection and characterization of molecular epitopes of lesser abundance, such as $\alpha_B\beta_3$-integrin, in early atherosclerosis. As powerful serum markers for the generalized state of inflammation become widely available for clinical use (eg, C-reactive protein), high-resolution in vivo molecular imaging methods could provide adjunctive data to precisely localize and quantify early or unstable vascular lesions that can be triaged rationally to medical treatment and then followed up serially for therapeutic response. Furthermore, as recently reported,\textsuperscript{22} this ligand-targeted nanoparticle system might carry a variety of drugs in its lipid membrane, rendering it an intriguing candidate for simultaneous molecular imaging and site-directed drug delivery for early atherosclerotic disease.

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