Novel MRI Contrast Agent for Molecular Imaging of Fibrin
Implications for Detecting Vulnerable Plaques

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Background—Molecular imaging of thrombus within fissures of vulnerable atherosclerotic plaques requires sensitive detection of a robust thrombus-specific contrast agent. In this study, we report the development and characterization of a novel ligand-targeted paramagnetic molecular imaging agent with high avidity for fibrin and the potential to sensitively detect active vulnerable plaques.

Methods and Results—The nanoparticles were formulated with 2.5 to 50 mol% Gd-DTPA-BOA, which corresponds to >50 000 Gd\(^{3+}\) atoms/particle. Paramagnetic nanoparticles were characterized in vitro and evaluated in vivo. In contradistinction to traditional blood-pool agents, T1 relaxation rate as a function of paramagnetic nanoparticle number was increased monotonically with Gd-DTPA concentration from 0.18 mL·s\(^{-1}\)·pmol\(^{-1}\) (10% Gd-DTPA nanoparticles) to 0.54 mL·s\(^{-1}\)·pmol\(^{-1}\) for the 40 mol% Gd-DTPA formulations. Fibrin clots targeted in vitro with paramagnetic nanoparticles presented a highly detectable, homogeneous T1-weighted contrast enhancement that improved with increasing gadolinium level (0, 2.5, and 20 mol% Gd). Higher-resolution scans and scanning electron microscopy revealed that the nanoparticles were present as a thin layer over the clot surface. In vivo contrast enhancement under open-circulation conditions was assessed in dogs. The contrast-to-noise ratio between the targeted clot (20 mol% Gd-DTPA nanoparticles) and blood was \(\approx 118 \pm 21\), and that between the targeted clot and the control clot was 131 \(\pm 37\).

Conclusions—These results suggest that molecular imaging of fibrin-targeted paramagnetic nanoparticles can provide sensitive detection and localization of fibrin and may allow early, direct identification of vulnerable plaques, leading to early therapeutic decisions. (Circulation. 2001;104:1280-1285.)

Key Words: magnetic resonance imaging ■ contrast media ■ plaque ■ fibrin

Since the early work of Benson\(^1\) and Constantinides,\(^2\) the acute formation of thrombus after atherosclerotic plaque rupture has been well recognized as the cause of unstable angina, myocardial infarction, transient ischemic attacks, and stroke. Although numerous medical advances in the detection and treatment of severe carotid and coronary artery stenosis have emerged, the most common source of thromboembolism remains rupturing of vulnerable plaques that reside in vessels with only 50% to 60% residual stenosis.\(^3,4\) Sensitive detection and differentiation of vulnerable versus stable atherosclerotic plaques in vessels with mild-severity stenoses remains limited with routine angiography or duplex ultrasound. These imaging modalities provide minimal information about arterial wall pathology. Moreover, compensatory arterial remodeling to preserve lumen dimensions within diseased vessels further disguises the severity of atherosclerotic plaque burden.\(^5\)

A variety of approaches to detect vulnerable plaques have arisen, based on intravascular ultrasound elastography,\(^6\) radionuclide imaging,\(^7\) and thermography,\(^8\) but MRI has emerged as a particularly sensitive modality to noninvasively visualize thromboses within the carotid artery.\(^9\) Recently, high-resolution MRI detection and characterization of atherosclerotic lesions (including advanced lesions, such as the fibrous cap, the lipid core, and even plaque fissuring) with serial imaging over time has been used to assess lesion progression or regression.\(^10,11\) High-resolution MRI can be used to distinguish intact thick fibrous caps from intact thin and disrupted caps in atherosclerotic human carotid arteries in vivo.\(^12,13\) Advances in high-spatial-resolution black-blood techniques may further improve noninvasive imaging of human coronary and carotid arteries and facilitate the early assessment of atherosclerotic disease.\(^14\)

We have developed a novel fibrin-specific MR contrast agent that could allow enhanced, sensitive detection and quantification of occult microthrombi within the intimal surface of atherosclerotic vessels in symptomatic patients and...
provide direct evidence to support acute therapeutic intervention. This unique agent is a ligand-directed, lipid-encapsulated liquid perfluorocarbon nanoparticle (250 nm nominal diameter) that has high avidity, has a prolonged systemic half-life, and can carry high Gd-DTPA payloads for high detection sensitivity.

We have recently defined the capability of this agent to detect fibrin clots in vitro with sizes from 0.5 to 7.0 mm with high-resolution MRI at 4.7 T. We have also reported that these ligand-targeted paramagnetic nanoparticles can be administered systemically in animal models and within 1 hour localize to the molecular signatures of angiogenesis, such as the expression of \( \alpha_\beta_3 \) integrin on neovascular endothelium. The objective of the present study was to demonstrate the potential of a fibrin-targeted, paramagnetic nanoparticulate contrast agent to specifically image fibrin deposits both in vitro and in vivo (in situ) at a clinically relevant magnetic field strength (1.5 T). We evaluated the capability of the paramagnetic nanoparticles to transport high payloads of gadolinium and estimated T1 relaxivities relative to particle number, reflecting their utility as a targeted molecular imaging rather than a blood-pool agent. The influence of in-plane resolution on the detectability and characterization of the paramagnetic particles is examined, and we demonstrated in vivo contrast enhancement under open-circulation conditions in dogs.

Methods

Preparation of a Biotinylated Perfluorocarbon Microemulsion for In Vitro Studies

The biotinylated perfluorocarbon contrast agent was produced in vitro studies by incorporating biotinylated phosphatidylethanolamine-4-\( \text{p} \)-maleimidophenylbutyramide (MPB-PE; Avanti Polar Lipids) into the outer lipid monolayer of a perfluorocarbon microemulsion. The surfactant comixture included various amounts of gadolinium DTPA-bis-oleate (Gd-DTPA-BOA) at overall concentrations of 0, 2.5, 5, 10, 20, 30, 40, and 50 mol\% of the outer lipid monolayer were diluted in fresh human blood. A Look-Locker sequence (TE/TR/\( \alpha \): 3.5/3000/10\(^{\circ}\)) with a coil aspect ratio of 128 \( \times \) 128 interpolated to 256 \( \times \) 256, field of view (FOV) 340 mm, image spacing 30 ms was used to measure longitudinal relaxation times. After a 180\(^{\circ}\) pulse, 50 gradient-echo images were acquired with a phase interval of 30 ms. All measurements were replicated twice. Images of the various emulsions were analyzed by defining regions of interest in each probe. Signal-intensity time curves for each phantom were obtained and fitted to the predicted magnetization curves.

Preparation of Ligand-Conjugated Acoustic Perfluorocarbon Emulsion Nanoparticles

The perfluorocarbon nanoparticle contrast agent used in vivo (in situ) was produced by incorporating 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-\( \text{p} \)-maleimidophenylbutyramide (MPB-PE; Avanti Polar Lipids) into the outer lipid monolayer of the emulsion to accommodate subsequent ligand conjugation.

Preparation, Isolation, and Conjugation of F(ab)\( _2 \) Fragments With MPB-PE Derivatized Emulsion

Anti-fibrin monoclonal antibody (NIB 1H10, NIB 5F3) was produced, purified, and biotinylated by conventional methods. A “1-step” fibrin-targeted nanoparticle contrast agent was created by the covalent bonding of anti-fibrin F(ab)\( _2 \) fragments to the outer lipid membrane surface. Anti-fibrin F(ab)\( _2 \) fragments were generated (Pierce) and combined with the MPB-PE–derivatized emulsion [1 to 2 mg F(ab)\( _2 \)/mL of 40% perfluorocarbon emulsion] at pH 6.7 under nitrogen overnight. The conjugated nanoparticles were dialyzed, placed into vials, and stored at 4°C. A nonspecific control emulsion was prepared by use of irrelevant IgG F(ab)\( _2 \) fragments.

Preparation of Human Plasma Clots for In Vitro Studies

Plasma clots were produced by combining plasma with 100 mmol/L CaCl\( _2 \) (3:1 vol/vol) and 5 U thrombin (Sigma). Thrombi were exposed serially to excess biotinylated antibody (25 \( \mu \)g/mL, 1 hour), excess avidin (25 \( \mu \)g/mL, 30 minutes, Pierce), and biotinylated emulsion (50 \( \mu \)L/mL, 30 minutes), with thorough washout of reactants between each step.

Scanning Electron Microscopy of Fibrin-Targeted Nanoparticles Bound to Fibrin Clots

Scanning electron microscopy was performed with a Philips scanning electron microscope. Fibrin-targeted and control clots were fixed in 2% glutaraldehyde, rinsed, and postfixed in 2% osmium tetroxide. Clots were dehydrated and treated with hexamethyldisilazane (Electron Microscopy Sciences).

MRI Experiments

All imaging experiments were performed with a 1.5-T clinical scanner (Gyroscan NT, Powertrak 6000, Philips Medical Systems) with a standard surface coil (6 cm).

Measurement of Nanoparticle T1 Relaxivities

Nanoparticles with Gd-DTPA-BOA incorporated at 0, 2.5, 5, 10, 20, 30, 40, and 50 mol% of the outer lipid monolayer were diluted in fresh human blood. A Look-Locker sequence (TE/TR/\( \alpha \): 10/70/90\(^{\circ}\)), acquisition matrix 128 \( \times \) 128 interpolated to 256 \( \times \) 256, field of view (FOV) 340 mm, image spacing 30 ms was used to measure longitudinal relaxation times. After a 180\(^{\circ}\) pulse, 50 gradient-echo images were acquired with a phase interval of 30 ms. All measurements were replicated twice. Images of the various emulsions were analyzed by defining regions of interest in each probe. Signal-intensity time curves for each phantom were obtained and fitted to the predicted magnetization curves.

MRI of Targeted Clots In Vitro

Two imaging experiments of the fibrin-targeted clots were performed. Plasma clots targeted with the paramagnetic nanoparticles at levels of 0, 5, 10, 20, and 40 mol% were immersed in plasma and imaged with a 3D T1-weighted gradient-recalled-echo sequence (TE/TR/\( \alpha \): 3.5/3000/10\(^{\circ}\)), acquisition matrix 128 \( \times \) 128 interpolated to 256 \( \times \) 256, field of view (FOV) 340 mm, image spacing 30 ms was used to measure longitudinal relaxation times. After a 180\(^{\circ}\) pulse, 50 gradient-echo images were acquired with a phase interval of 30 ms. All measurements were replicated twice. Images of the various emulsions were analyzed by defining regions of interest in each probe. Signal-intensity time curves for each phantom were obtained and fitted to the predicted magnetization curves.

In Vivo In Situ Canine Thrombus Preparations

The concept of the fibrin-targeted, paramagnetic nanoparticles to enhance the detectability of clots in a flowing intravascular environment was evaluated in dogs. Thrombi were formed within the open circulation, targeted with a single-step system in situ within isolated vascular segments, then exposed to the systemic circulation for MRI. Animal protocols were approved by the Animal Studies Committee at Washington University.

Two dogs (\( \sim \)20 kg) were pretreated with tranexamic acid (0.25 g/h) to inhibit endogenous thrombolysis. Each animal was anesthe-
tized (sodium pentathol/isoflurane) and prepped for surgery, and the external jugular veins were exposed. Nylon monofilament (4-0) with ten 0.5-cm strands of thrombin-soaked cotton fibers were positioned by ultrasound (Acuson Sequoia). After clot formation, thrombi were entrapped between snare closures, and 1 mL of fibrin-targeted gadolinium or control nanoparticles was infused into the isolated segment. After contrast incubation (1 hour), the thrombi were reintroduced to the general circulation and imaged. At the conclusion of the acute procedure, animals were euthanized and the vessels retrieved for routine immunohistopathology of fibrin within the thrombus.

In Vivo MRI
Canine thrombi created within the external jugular vein were imaged with a 3D, fat-suppressed, T1-weighted fast gradient echo (TE/TR/α: 8.1/24/35°, FOV 180 mm, matrix 205×256). Flow within vessels and thrombi (as a flow deficit) were imaged with a 3D phase-contrast, T1-weighted fast-gradient-echo angiogram (TE/TR/α: 5.3/15/15°, FOV 200 mm, matrix 192×256).

Results
Scanning electron micrographs of fibrin clots exposed to control and fibrin-targeted nanoparticles revealed a tight weave of fibrils, which precluded deep penetration of the nanoparticles (Figure 1). Fibrin-targeted nanoparticles densely and specifically adhered to the clot surface, with each bound complex delivering tens of thousands of Gd-DTPA molecules. The targeted nanoparticles create a very thin, gadolinium-rich layer that encases the clot surface. The classic description of relaxivity with respect to the absolute Gd-DTPA concentration used for blood-pool agents can be misleading for targeted paramagnetic agents, which depend on the payload of gadolinium delivered to each molecular epitope. Accordingly, we estimated T1 relaxation rate as a function of the nanoparticle number (Figure 2), which increased monotonically with increasing Gd-DTPA-BOA (Figure 3). From these data, particle-specific relaxivity in units of mL·s⁻¹·pmol⁻¹ were calculated and increased monotonically from 0.18 mL·s⁻¹·pmol⁻¹ for the 10% Gd-DTPA-BOA nanoparticles (10 000 Gd³⁺/particle) to 0.54 mL·s⁻¹·pmol⁻¹ for the 40 mol% Gd-DTPA-BOA (50 000 Gd³⁺/particle) formulations.

MRI demonstrated the significant contrast enhancement produced by the fibrin-specific nanoparticles targeted to human plasma clots in vitro at a clinically relevant field strength (1.5 T). With a typical low-resolution clinical imaging protocol, the fibrin clots targeted with nanoparticles presented a homogeneous T1-weighted contrast enhancement that improved with increasing gadolinium level (0, 2.5, and 20 mol% Gd) (Figure 4a). Higher-resolution scans (Figure 4b) and scanning electron microscopy (Figure 1), however, indicated that the nanoparticles were present only in a thin outer layer. The large number of nanoparticles bound, in conjunction with the high payloads of gadolinium each carries, diminished the partial-volume, signal-dilution effect of the low-resolution voxel. The contrast-to-noise ratio (CNR) between the targeted 20 mol% Gd-DTPA-BOA clot and surrounding blood measured with the 3D GraSE sequence was 60±8, and that between the targeted (20 mol% Gd-DTPA-BOA) and the control clot was 75±15. Peak signal along the clot surface increased linearly as in-plane voxel size decreased from 0.4 to 0.1 mm and as nanoparticle Gd-DTPA-BOA payload increased from 0 to 40 mol% of the contrast agent without reaching a plateau (Figure 4b).

The magnitude of contrast enhancement expected in vivo with open-circulation conditions was evaluated in dogs. Control or 20 mol% Gd-DTPA-PE anti-fibrin nanoparticles were targeted in situ as a single-step system to thrombus created within the external jugular vein. Thrombus was imaged with a 3D T1-weighted, fat-suppressed, fast-gradient-echo sequence, and the detectability of targeted clot was markedly enhanced by the fibrin-specific paramagnetic nanoparticles relative to control thrombus (Figure 5a and 5b). Phase-contrast angiography revealed the clots as flow deficits in both external jugular veins. Corresponding gradient-echo

Figure 1. Scanning electron micrographs (>×30 000) of control fibrin clot (A) and fibrin-targeted paramagnetic nanoparticles bound to clot surface (B). Arrows indicate (A) fibrin fibril; (B) fibrin-specific nanoparticle-bound fibrin epitopes.

Figure 2. T1-relaxation rates as a function of nanoparticle number expressed in pmol for formulations ranging from 0 to 50 mol% Gd-DTPA-BOA in outer 2% lipid monolayer.
images revealed a selective enhancement of the treated clot, yielding a signal intensity (1780 ± 327) higher than the bright fat signal (1360 ± 140), whereas the control clot had a signal intensity (815 ± 41) similar to that of the adjacent muscle (768 ± 47). On T1-weighted gradient-recalled-echo images with fat suppression, the targeted clot showed the brightest image signal. The CNR between the targeted clot and blood when nanoparticles with 20 mol% Gd-DTPA measured with this sequence were used was ≈118 ± 21. The CNR between the targeted clot and the control clot was 131 ± 37. Fibrin immunostaining of the excised vessel and clot confirmed the abundance and localization of fibrin corresponding to the contrast enhancement in vivo.

Discussion
Recent studies reveal that vulnerable plaque rupture and microthrombus formation precede acute myocardial infarction by days to months, providing an opportunity to intercede and prevent serious sequelae. Sensitive molecular imaging and detection of microthrombi along the intimal surface of vulnerable plaques require a high-avidity, clot-specific MR contrast agent with high paramagnetic impact. Each nanoparticle bears an estimated 50 to 100 Fab fragments targeted, imparting very high avidity. Moreover, we have reported that the nanoparticles have a prolonged systemic half-life of ≈1 hour, which allows them to reach and enhance the detection of molecular epitopes, as recently demonstrated by the detection of αvβ3 integrin on angiogenic vessels.

In this report, we demonstrate that nanoparticles can bear enormous paramagnetic payloads, provide a high T1-weighted contrast signal, and overcome the dilutional partial-volume effects that have caused most MRI targeted contrast agent formulations to fail in vivo. With high-resolution MRI (4.7-T), fibrin clots as small as 500 μm can be clearly detected and morphologically delineated. In the present study, similar clots imaged at 1.5 T are detectable, with an expected loss in morphological resolution. We have shown that a thin surface layer of paramagnetic nanoparticles (200 nm nominal) can dramatically enhance the detection of fibrin deposits.

Molecular probes must have high specificity, provide marked signal amplification, and be compatible with high-resolution imaging systems. The magnitude of targeted

Figure 3. Signal-to-noise ratio of fibrin-specific contrast enhancement on 3D T1-weighted gradient-recalled-echo images as a function of increasing Gd-DTPA-BOA concentration in outer 2% lipid monolayer.

Figure 4. A, Low-resolution images (3D GraSE) of fibrin clots targeted with nanoparticles presenting a homogeneous, T1-weighted enhancement that improves with increasing gadolinium level (0, 2.5, and 20 mol%). B, High-resolution scans of fibrin clots (3D T1-weighted gradient-recalled-echo sequence) revealing thin layer of nanoparticles along surface. Peak signal increases with increasing gadolinium concentration (5, 10, 20, and 40 mol%) and decreasing voxel size (0.4, 0.2, and 0.1 mm).

Figure 5. A, Thrombi in external jugular vein targeted with fibrin-specific paramagnetic nanoparticles demonstrating dramatic T1-weighted contrast enhancement in gradient-echo image (arrow) on left with flow deficit (arrow) of thrombus in corresponding phase-contrast image on right (3D phase-contrast angiogram). B, Control thrombus in contralateral external jugular vein imaged as in A.
contrast achieved reflects the number and density of nanoparticles bound and the paramagnetic influence that each particle exerts (ie, gadolinium payload). In molecular imaging applications, the paramagnetic influence of each binding complex becomes critically important. Depending on the formulation applied, the nanoparticles can deposit >50 000 gadolinium atoms at each receptor site. Calculating R1 relaxivity per picomole of nanoparticles provides a unique estimate of the contrast effect expected by each binding complex. Although many of the new dendrimeric contrast agents have high relaxivity and are excellent blood-pool agents,28 these paramagnetic complexes when used for molecular imaging bring orders of magnitude less gadolinium to each targeted epitope than nanoparticles. Moreover, their relatively low avidity for targets and short systemic longevity have posed additional barriers to overcome. The nanoparticles provide little or no blood-pool contrast when administered in vivo (0.25 to 0.5 mL/kg), but when they bind and collect at a targeted site, such as a thrombus, their T1-weighted contrast effects are substantial. Thus, they inherently have high signal-to-noise ratios.

Increasing nanoparticle Gd-DTPA payload increased the T1-weighted contrast effect without reaching a saturation plateau. At very high concentrations, however, Gd-DTPA-BOA formulations appeared to be less stable. Substitution of Gd-DTPA-BOA with an alternative lipophilic gadolinium chelate, Gd-DTPA-PE,29 has provided a more stable formulation in preliminary evaluation. Recent data further suggest that nanoparticle formulations of Gd-DTPA-PE have twice the R1 relaxivity versus equimolar Gd-DTPA-BOA nanoparticles.30

Although we used an avidin-biotin–based nanoparticle targeting approach for in vitro studies, clinical applications demand a simpler, single-step system as used in the present study to target thrombi in dogs. Nanoparticle-targeted thrombi within the external jugular veins of 2 dogs demonstrated higher signal intensities than the bright fat signals, whereas the control clots had signal intensities similar to those of the adjacent muscle. Substantial CNRs between the targeted clot and blood or between the targeted and the control clots were obvious from the images themselves. These results demonstrate that fibrin-targeted paramagnetic nanoparticles can markedly enhance the detectability of partially occlusive thrombi under in vivo conditions.

As a further conceptual example, carotid artery endarterectomy specimens from symptomatic patients were exposed to either targeted or control paramagnetic nanoparticles and imaged with a T1-weighted GraSE sequence similar to that used in the canine experiments (Figure 6). The enhancement of the small fibrin deposits in the ruptured carotid plaque treated with targeted paramagnetic nanoparticles was readily apparent, in contradistinction to the control specimens. Similar direct diagnosis of intimal fibrin deposition in a patient with moderate carotid stenosis may someday prompt early surgical or interventional therapy.

In summary, we report a novel fibrin-targeted paramagnetic molecular imaging system that enhances the sensitive detection of clots in vitro and in vivo. This unique agent, because of its strong MR contrast impact, has the ability to enhance the detection of intravascular clots and minute thrombi within fissures of active vulnerable plaques. We suggest that emerging molecular imaging technologies, such as these paramagnetic nanoparticles, may provide early direct diagnosis of impending stroke or infarction in patients presenting with heralding symptoms and support early therapeutic intervention.

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References


Figure 6. Color-enhanced MR images of fibrin-targeted and control carotid endarterectomy specimens revealing contrast enhancement (white) of small fibrin deposit on symptomatic ruptured plaque. Calcium deposit (black). 3D, fat-suppressed, T1-weighted fast gradient echo. NP indicates nanoparticle.
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