Molecular Imaging in Atherosclerosis, Thrombosis, and Vascular Inflammation

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Abstract—Appreciation of the molecular and cellular processes of atherosclerosis, thrombosis, and vascular inflammation has identified new targets for imaging. The common goals of molecular imaging approaches are to accelerate and refine diagnosis, provide insights that reveal disease diversity, guide specific therapies, and monitor the effects of those therapies. Here we undertake a comparative analysis of imaging modalities that have been used in this disease area. We consider the elements of contrast agents, emphasizing how an understanding of the biology of atherosclerosis and its complications can inform optimal design. We address the potential and limitations of current contrast approaches in respect of translation to clinically usable agents and speculate on future applications. (Arterioscler Thromb Vasc Biol. 2009;29:983-991.)

Key Words: atherosclerosis ■ inflammation ■ molecular imaging

Appreciation of the molecular and cellular processes of atherosclerosis, thrombosis, and vascular inflammation opens the way for commensurately sophisticated approaches to disease characterization through imaging.1–6 The common goals of molecular imaging approaches are to accelerate and refine diagnosis, provide insights that reveal disease diversity, guide specific therapies, and monitor the effects of those therapies. To these ends, a range of contrast methodologies are in development across a number of modalities. This review will undertake a comparative analysis of imaging modalities applicable to atherosclerosis, thrombosis, and vascular inflammation and highlight some of the molecular, cellular, and functional targets that show greatest potential. It will compare the attributes of different approaches and relate these to specific applications, emphasizing the opportunities and challenges for each. Other reviews in this series will provide detailed systematic consideration of individual modalities.

As the repertoire of molecular contrast agents expands and more show potential in proof of principle studies, we will consider the routes and obstacles to the development of agents that might be used in the clinical setting.

Targets

Vascular disease is relatively privileged compared, for instance, to neurological imaging because many of the targets are accessible to the blood and blood-borne reagents. There are also specific impediments. Blood vessels are often deeply located structures, which can restrict the application of low penetration techniques, such as fluorescence imaging or ultrasound unless intravascular imaging systems are developed. In addition, atherosclerotic plaques are relatively small structures and there are challenges of movement because of cardiac and respiratory motion. High shear stresses of blood in large arteries can be challenging to particulate contrast agents.

To highlight potential imaging targets, it may be helpful to consider atherogenesis in terms of (1) early processes; (2) progression to more advanced lesions; and (3) thrombotic complications. Specific imaging targets, discussed below, are highlighted in Figure 1.

Events in Early Atherogenesis

Early in atherogenesis, disordered endothelial function accelerates the deposition of apolipoprotein B–containing lipoprotein particles in the subendothelial space.7 A fraction of these particles are retained, which promotes local inflammation, characterized by the release of soluble signaling factors, including chemokines,8 and by the expression of endothelial cell adhesion molecules, eg, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P-selectin,9–11 which recruit mononuclear leukocytes, especially monocytes and T-lymphocytes, to the arterial wall.12 Recruited monocytes differentiate into macrophages and upregulate several scavenger receptors capable of binding modified forms of low density lipoproteins (LDL) including scavenger receptor types AI and AII (SR-AI, SR-AII),

Received December 31, 2008; revision accepted February 3, 2009.
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© 2009 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBAHA.108.165498

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CD36, CD68, LOX-1, and SR-PSOX/CXCL16. Modified LDL taken up via scavenger receptors is delivered to lysosomes, where enzymes hydrolyze cholesteryl esters to free cholesterol and fatty acids. Inside macrophages, the enzyme acyl-CoA:cholesterol acyltransferase (ACAT) catalyzes the formation of cholesteryl ester, which accumulates in characteristic foamy deposits.

**Progression of Atherosclerosis**

The net rate of accumulation of cholesterol in the plaque reflects the difference between its rate of deposition and extraction from the plaque by high density lipoproteins. High local concentrations of cholesterol may be associated with apoptosis and necrosis of plaque macrophages. When intracellular storage capacity is exceeded, free cholesterol is able to accumulate in the extracellular domain. Ruptured plaques, which underlie acute atherothrombotic events, commonly contain high concentrations of macrophages, particularly in the superficial “shoulder” region. Plaque macrophages secrete matrix metalloproteinases that digest the stabilizing connective tissue elements of plaque promoting vulnerability to rupture. In addition to destabilizing plaque, macrophages secrete prothrombotic tissue factor, which accelerates thrombus formation after rupture or erosion. However, because MMP activity is regulated (by tissue inhibitors of metalloproteases or TIMPs), it may not be sufficient to image the mere presence of MMPs. More meaningful insights will be derived from assessment of the proteolytic activity of MMPs within the plaque, analogous to in situ zymography.

**Thrombotic Complications**

Rupture or erosion of an atherosclerotic plaque is associated with local deposition of activated platelets, fibrin, and tissue factor. The ability to localize, quantify, and characterize acute thrombus rapidly and noninvasively would be of great clinical utility in acute vascular syndromes and could inform the most appropriate choice of therapy.

**Generic Requirements for Molecular Imaging**

Conceptually, molecular imaging requires multiple functional components, though in practice more than one function can be integrated into a single element. Localization and retention at the site of interest is typically via (1) a ligand with both affinity and specificity for the target (e.g., antibody, antibody fragment, peptide, aptamer or oligosacharide). A contrast or (2) signaling element (e.g., iron oxides, gadolinium, radionuclides, fluorochromes, gas-filled microbubbles) may be incorporated directly into the ligand or may be conveyed by a (3) carrier vehicle (e.g., micelle, perfluorocarbon nanoparticle, synthetic lipoprotein particle, polymer-derived microparticle, carbon nanotube, Figure 2). Determinants of accessibility and mechanisms of conveyance to the target (e.g., diffusion, mass flow, receptor-mediated internalization, pinocytosis, intracellular carriage) will depend on the (4) physical and chemical properties the contrast agent (e.g., size, charge, hydrophobic/hydrophilic exterior, specific surface molecules). Design-for-purpose of a contrast agent or class of agents requires consideration and optimization of these multiple elements.

**Ligand**

A ligand should possess both specificity and affinity for the target of interest. In experimental studies, antibodies have...
been used against numerous targets that include VCAM-1, P-selectin, ICAM-1, αβ₃ integrin, epitopes on oxidized LDL, and macrophage scavenger receptor. Thus antibodies have proven effective despite potential steric limitations imposed by their relatively large size. Potential immunogenicity can be attenuated by the use of modified or “humanized” antibodies and by the use of single chain antibodies. However, antibody production is also relatively complex and expensive to generate in a quantity that would be required for use as a clinical reagent. Alternative approaches have used peptides, such as the RGD motif that binds to integrins, and oligosaccharides. In a recent novel approach, synthetic structures, incorporating proteins with posttranslational modifications, have been generated to achieve functional mimicry of naturally occurring ligands, eg, P-selectin glycoprotein ligand, and have targeted vascular inflammation.

Multiple Ligands
In large and medium arteries that are affected by atherosclerosis, high shear stresses can impair receptor-ligand interactions. Low-affinity interactions are potentially enhanced by polyvalent ligand presentation, which may be accomplished by conjugating the ligand of interest to microparticles. Physiologically, leukocytes are arrested at sites of inflammation by using multiple receptor ligand interactions. Using combinations of ligand pairings (eg, antibodies against VCAM-1 and P-selectin or antibody to ICAM-1 and sialyl-Lewis X, targeting selectins) on the surface the same particles, binding can be enhanced by up to an order of magnitude compared to binding mediated by single interaction binding. Retention at target can be further amplified by strategies that result in local accumulation. For example, a high-specificity probe targeting VCAM-1 and internalized by endothelial cells after binding has been developed through phage display. In this way, ligand characteristics can be used to enhance sensitivity for imaging the endothelial monolayer, a cell-type that is of crucial importance but low abundance compared to other cell types of the arterial wall.

Phage Display
Phage display is a powerful, if labor-intensive, technique that allows a huge range of potentially useful diagnostic and therapeutic antibodies to be evaluated in a functionally relevant context—and for the useful ones to be produced in large quantities. Bacteria and bacterial viruses, known as phage, are used to produce synthetic antibodies with highly diverse target recognition (“variable”) regions based on the corresponding DNA (cDNA) obtained from reverse transcription of mammalian B-lymphocyte total messenger RNA (mRNA). The phage are engineered so that the antibody is fused to a protein that is expressed on the phage coat and the gene encoding for that specific antibody is contained within the coat. Collections of the antibody-coated phage (typically in the order of 10⁹ separate permutations) constitute a phage library. Those of interest can be selected for by solid phase immunosorption mediated by the surface antibody and amplified once isolated. In this way, further ligands have been identified that target cell adhesion molecules and activated platelets via the activate conformation of glycoprotein IIbIIIa (Figure 3). In another variation of phage display, instead of antibodies, peptides are expressed on the phage coat, and those that interact with receptors or other surface molecules on the target cells can be similarly identified and enriched.
Kinetics
The on–off kinetics of ligand binding are important. Clinically useful reagents are likely to display relatively rapid “on” kinetics that allow imaging soon (minutes to hours) after contrast administration. Although this may be feasible for imaging intravascular targets such as endothelial adhesion molecules or components of thrombus, delivery to targets within the plaque is likely to take longer (hours to days). To enable sequential imaging in determining disease activity and response to treatment, swift removal and clearance of the contrast agent are desirable characteristics. Most molecular contrast agent studies to date report single time point imaging that provides limited insight into the “on-kinetics” and often no evaluation of tissue residency, redistribution, or disposal. If data are to be useful in quantification of the target of interest, the kinetics need to be reproducible and predictable.

Contrast or Signaling Agent

Optical Imaging
Molecular imaging modalities vary intrinsically in their sensitivities. Fluorescence techniques possess high sensitivity, but are limited by tissue penetrance, including in blood. Fluorescent probes that can be incorporated into relatively small molecules that possess, or retain, biological function bring accessibility, combined with a functional versatility that may be lacking where larger carrier vehicles are required (e.g., micelles, liposomes, lipoproteins; see below). Use of multiple fluorochromes targeting different molecules opens the way for multiplex imaging to provide insights of greater complexity. Besides reporting simple accumulation, polymeric probes can be constructed in which fixed close proximity of fluorochromes limit their capacity for excitation, such that fluorescence is quenched before specific enzymic cleavage that liberates the fluorescent capability. In this way a level of functional specificity is generated. Examples in vascular imaging include mapping the activity of the macrophage-associated enzymes cathepsins B and K in mouse atherosclerosis and matrix metalloproteinases. The precise depth of tissue penetrance varies with the fluorochrome and the tissue under evaluation. Although quantitative depth-resolved NIR fluorescence systems are routinely available for mice, there are limitations in the assessment of deep structures such as the coronary arteries in humans. As a result, this type of imaging is likely to make its greatest contributions as an experimental tool. Development of catheter-mounted fiber-optic devices for use in intravascular imaging offers promise for clinical application, particularly if this can be combined with a mode of intravascular anatomic imaging, such as intravascular ultrasound, optical coherence tomography, or MRI.

PET and SPECT
Radionuclides provide a high degree of sensitivity, with detection possible in the picomolar range. Single positron emission tomography (SPECT) has been used to image apoptosis, by targeting annexin A5 in rabbits and to track monocytes in experimental mouse atherosclerosis. To date, 18Fluoro-deoxyglucose (18FDG) has been most commonly used PET agent in vascular imaging. This modified glucose analogue is transported into cells that are active in glycolysis. However, inability to pass through the glycolytic pathway leads to intracellular accumulation. Imaging with 18FDG has shown initial promise in the aorta and carotid arteries, where its accumulation has been shown to correlate with macrophage infiltration, determined by immunohistochemistry in explanted carotid endarterectomy samples, and to change in response to treatment. Proximity to the highly metabolically active myocardium, and consequent signal spillover, limits use of 18FDG for coronary imaging. However, PET imaging is not limited to 18FDG, and there is an expanding range of radio-ligands.
A further appeal of this modality is the opportunity to radiolabel drugs and to quantify receptor binding and pharmacodynamics in vivo. The lack of anatomic detail from PET images, can be largely addressed by coregistration with either CT or MRI.

Of approaches to molecular imaging, PET and SPECT are the only ones that are established in clinical use, though there are also important logistical drawbacks. As radioisotopes, the contrast reagents are expensive and are not stable for more than short periods. Furthermore access is relatively limited both to PET scanners and radionuclide sources.

**Magnetic Resonance Imaging**

By comparison, MRI provides a high degree of spatial resolution and soft tissue contrast yielding excellent anatomic detail, but is of inherently low sensitivity for molecular imaging applications. In MRI, appearances are determined by the density and local environment of water protons. MR contrast agents exert indirect effects by altering the properties of these protons and their local environment. Compared to fluorescence or PET imaging, attainment of contrast in MRI requires the delivery of relatively large quantities of contrast agent to the site of interest (micromolar range). Typically, these agents comprise gadolinium ions chelated to small molecules (to attenuate toxicity of gadolinium ions). Gadolinium shortens T1, leading to bright appearance or positive contrast on T1-weighted images. Alternatively, superparamagnetic iron oxides confer greater sensitivity. These contrast agents induce proton dephasing, usually manifest as intensely negative contrast on T2*-weighted images. The range of iron-based contrast agents and methods for obtaining “positive contrast” effects have recently been reviewed comprehensively. Importantly, for enhancing sensitivity in molecular imaging applications, the contrast effects derived from iron oxides can manifest in a volume that is orders of magnitude greater than the physical size of the particles that contain them.

Shapiro et al have shown how micron-size range (0.9 to 8.5 μm) particles containing iron oxide can exert contrast effects that extend ~50 times the physical dimension of the particle. Initially used in cellular imaging applications, microparticles have more recently been applied to molecular imaging in vascular inflammation, atherosclerosis, and platelet thrombosis (Figure 4). Further consideration of the different sizes and composition of iron oxide-containing particles is given below.

Analogous to the enzymatic cleavage sites incorporated into quenched-state fluorochromes, sophisticated MR agents have been engineered to conceal gadolinium from tissue water (thus quenching its contrast efficacy), unless a specific enzyme-mediated cleavage occurs. In this way, it has been possible to image β-galactosidase activity as a marker of transgene expression. Similarly, activity of the enzyme myeloperoxidase (released from activated neutrophils and macrophages, and elevated in blood in acute coronary syn-
Ultrasound

Ultrasound provides relatively low resolution anatomic data, but with the very great advantages of being widely available, cheap, and portable. Numerous investigators have adopted approaches in which molecular imaging has been attained through the use of targeted gas-filled micobubbles. These particles, such as liposomes or gas-filled phospholipid microbubbles, produce intense acoustic reflection. By this approach it has been possible to image a range of targets similar to those described for MRI (above). Specifically, ICAM-1, VCAM-1, P-selectin, fibrin, and integrins have all been targeted using ultrasound probes.

Carriers

For the high sensitivity modalities, eg, PET and fluorescence imaging, the contrast agent may simply comprise a small molecule. For lower sensitivity techniques eg, ultrasound and MRI, a carrier vehicle or amplification particle (eg, liposome, micelle, lipoprotein, carbon nanotube, dextran particle) may be required.

MRI

Nanometer-size-range iron oxide particles (ultrasmall particles of iron oxide, USPIO) can be used for cellular imaging purposes and have been used as passive contrast agents to identify plaque macrophages in humans. To provide a magnetic “shield” that minimizes aggregation, these particles have a polymeric coat surrounding a nucleus of iron oxides. Commonly the glucose-based polymer dextran is used, because it is relatively straightforward to manipulate and is biocompatible, including for use in humans. To add structural stability, cross-linked preparations (cross linked iron oxides, termed “CLIO”) have been developed with functionalized exteriors that permit covalent conjugation of surface ligands for molecular imaging purposes.

USPIO have a long half-life in the blood, which is a positive attribute for applications such as the measurement of tissue perfusion. However, this property is more of a hindrance in targeted contrast agents because it leads to high background contrast for an extended period. A further potential drawback of USPIO is that contrast is manifest in T2*-weighted images as indistinct areas of low signal that can be difficult to distinguish from the ordinary heterogeneity of normal tissue. Furthermore, because USPIO can be taken up nonspecifically and can extravasate passively, particularly in the leaky vessels associated with sites of inflammation, there is potential to compromise the specificity of molecular targeting. We have used much larger microparticles of iron oxide (0.9 to 4.5 μm; “MPIO”) for molecular imaging applications. The payload of iron is high and the contrast effect is pronounced. For imaging endovascular structures, MPIO possess several positive attributes. First, the particles convey a payload of iron that is many orders of magnitude greater than USPIO. Second, the effects of MPIO on local magnetic field homogeneity, and therefore detectable contrast, extend for a distance \( \approx 50 \) times the physical diameter of the microparticle. Third, unlike USPIO, the relatively large size of MPIO means that they are less susceptible to extravasation or nonspecific uptake by endothelial cells and therefore retain specificity for molecular targets expressed on the vascular endothelium. However, the corollary of this is an inability of MPIO to image epitopes found deeper within the plaque.

The ability to manipulate the physical properties and composition of contrast particles presents opportunities to harness physiological pathways. For instance, we have isolated and delipidated normal human HDL to obtain its apolipoproteins, mainly apolipoprotein (apo) A-I. The apolipoproteins can then be extracted and reconstituted with phospholipids, with or without unesterified cholesterol. To generate a MR contrast agent, phospholipid-based Gd-DTPA-DMPE is incorporated into the reconstituted particle of approximately 9 nm diameter. For confocal fluorescence microscopy studies, a fluorescent phospholipid with a green emission was also added. More recently, in some preparations, the Gd-DTPA-DMPE and fluorescent phospholipid have been added to intact HDL by gentle sonication. In either type of reconstruction, when intravenously injected, these small particles accumulate within atheromatous plaques, following HDL pathways into plaque macrophages (Figure 4). To avoid the requirement for human blood-derived HDL, we have developed an apoA-I–mimicking peptide, 37pA, to replace native apoA-I, thereby avoiding the lengthy separation procedure required to isolate HDL. Particles formed from 37pA have been reported to perform functions of native HDL, such as cholesterol efflux and binding to the ABCA-1 transporter and are effective imaging agents.

Ultrasound

Gas-filled microbubbles for ultrasound imaging comprise albumin, lipid, or perfluorocarbon shells around a gas-filled core, (typical size range 5 to 20 μm). The sensitivity of ultrasound is such that single static microbubbles can be detected with transcutaneous ultrasound. Furthermore, bubbles can be instantaneously destroyed by delivery of a sonic pulse, providing a way to confirm binding, an opportunity to interrogate the dynamics of binding and a method for local delivery of any encapsulated drugs.

Clearance

Anticipating potential clinical application, contrast clearance is an important consideration. In the first place, rapid blood phase clearance may be required to remove background signal to permit identification of retained contrast at the site of interest. Afterward, clearance from the target site will permit repeated measures, for example to monitor response to treatment. In the final phase, the agent should undergo disposal or excretion, with or without prior dismantling to component elements. For example it has been proposed that microparticulate contrast agents might be dismantled in the reticuloendothelial system with iron incorporation into the general pool. Variations in size and surface composition can influence routes and rates of disposal.

To date, though,
studies that have focused on proof of principle imaging have typically neglected detailed evaluation of effects of variable dosing, timing, and routes of disposal.

**Toxicity**

Clearly, a favorable safety profile is a prerequisite for clinical use, and the regulatory requirements for demonstration of safety are no less stringent for diagnostic contrast agents than for therapeutic agents. Areas requiring evaluation include immunogenicity, radioactivity, chemical toxicity, potential for pharmacological action, physical toxicity (eg, vessel plugging), potential for accumulation on repeated dosing.

In general little is known of these, and studies tend to be small scale, organ- or system-specific with a lack of dose ranging evaluation, effect of repeat administrations, time course, or anything other than immediate toxicology. As candidates for translation to human emerge, evaluation of these parameters will be vital.

**Therapeutic Options**

Delivery of targeted agents that can be localized to areas of disease and quantified presents clear opportunities for efficient delivery of site-specific therapies.\(^5\) In vascular disease, these might include agents that target endothelial function, inflammation, angiogenesis, cellular proliferation, and coagulation.

Reduced neointimal formation in a rabbit model of in-stent restenosis has been accomplished using systemically delivered albumin-nanoparticles containing paclitaxel.\(^6\) Recently reported was the use of nanoparticle delivery of prednisolone, encapsulated in liposomes targeting chondroitin sulfate proteoglycans in the vessel wall, which are exposed after stent implantation.\(^7\) A similar approach has incorporated the antiproliferative agents doxorubicin and paclitaxel in to nanoparticles targeting tissue factor. Coloaded with gadolinium, these particles showed demonstrable T1 effects with MR at 4.7T.\(^8\) Moreover, by using \(^19\)F incorporated into perfluorocarbon nanoparticles, these authors propose a method for direct spectroscopic quantification of particle accumulation. An elegant approach to drug delivery using imaging particles has involved incorporation of the lipid soluble antiangiogenic compound fumagallin into nanoparticles targeted to \(\alpha_v\beta_{III}\) integrin, expressed in the neovascularure of rabbit atherosclerosis.\(^8\) Future iterations of combined therapeutic/diagnostic agents may benefit from advances in polymer science that will enable delivery of biologically inert particles that are spontaneously degraded without deleterious effects.\(^9\)

**The Future and Clinical Application**

Future technical developments will focus on each of the components identified above with specific interest in the development of more sophisticated targeting strategies and the use of multimodal imaging probes that will combine sensitivities to make best use of integrated MRI and PET. Already, there has been much progress in the area of molecular imaging for vascular diseases. Yet the hurdles to translation into the clinical realm are considerable. Although ultrasound and MRI molecular contrast agents have been used in humans, of the modalities discussed above, only PET and SPECT have molecular contrast agents in routine clinical use. A challenge to scientists and their commercial partners is how to pick the right agent at the right time in its development and to define and application to move forward to clinic.

**Sources of Funding**

Dr Choudhury’s laboratory is funded by the Wellcome Trust, the British Heart Foundation, and by the Oxford Comprehensive Biomedical Research Centre, NIHR funding scheme. Dr Fisher’s work in this area is funded by NIH grant R01HL078667.

**Disclosures**

None.

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Arterioscler Thromb Vasc Biol. 2009;29:983-991; originally published online February 12, 2009;
doi: 10.1161/ATVBAHA.108.165498
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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