

# Cellular and Molecular Imaging of Vulnerable Plaque

The potential of optical coherence tomography.

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**P**laque rupture leading to myocardial infarction (MI), cerebral vascular accident (CVA), and progression of peripheral artery disease (PAD) remains the leading cause of death in the world. Atherosclerosis accounts for 23% of all deaths worldwide, far surpassing both infectious diseases and cancer.<sup>1</sup> Recent evidence from many investigational studies clearly indicates that vessel occlusions are due to sudden plaque rupture of noncritically stenotic arteries and not due to progression of critically stenotic vessels, as thought previously. Little et al observed that approximately 70% of patients suffering from MIs were initiated on plaques that were >50% stenosis, as revealed by previous coronary angiography.<sup>2</sup> These observations have also been confirmed by other investigators.<sup>3-5</sup> Thus, lesions associated with noncritical stenosis are most likely to rupture and cause an MI or CVA. Because atherosclerosis is a diffuse disease involving large segments of the arterial intima, the entire luminal surfaces of the coronary, cerebral, and peripheral arteries are at risk.

Plaque that tends to rupture, also known as *unstable plaque*, was first identified and characterized by pathologists in the 1980s. Today, the principal pathologic features are well described and include a thin fibrous cap of >65 nm, increased infiltration of macrophages with decreased smooth muscle cells, and an increased lipid core size.<sup>6-9</sup> Macrophages are an important cellular marker and contribute to the risk of plaque rupture in the coronary, cerebral, and peripheral circulations. Activation and recruitment of macrophages can induce breakdown of a thin fibrous cap and increase mechanical instability and the risk of plaque rupture by local production of matrix metalloproteinases (MMPs). Greater macrophage density has been associated with increased plaque vulnerability.<sup>10</sup>

Understanding these molecular and cellular events has been the stimulus for developing new imaging approaches. We review these competing technologies (Figure 1) for cellular and molecular imaging of vulnerable plaque in this article.

## SPECTROSCOPY

Because different tissue types with unique combinations of carbon-hydrogen, carbon-oxygen, and other bonds have different but characteristic photon-spectral absorbance,<sup>11</sup> tissues can be differentiated and identified as unstable (lipid rich) as opposed to stable (fibrous rich).<sup>12</sup> Currently, the most validated spectroscopic approaches are Raman spectroscopy and near-infrared reflection (NIR) spectroscopy.

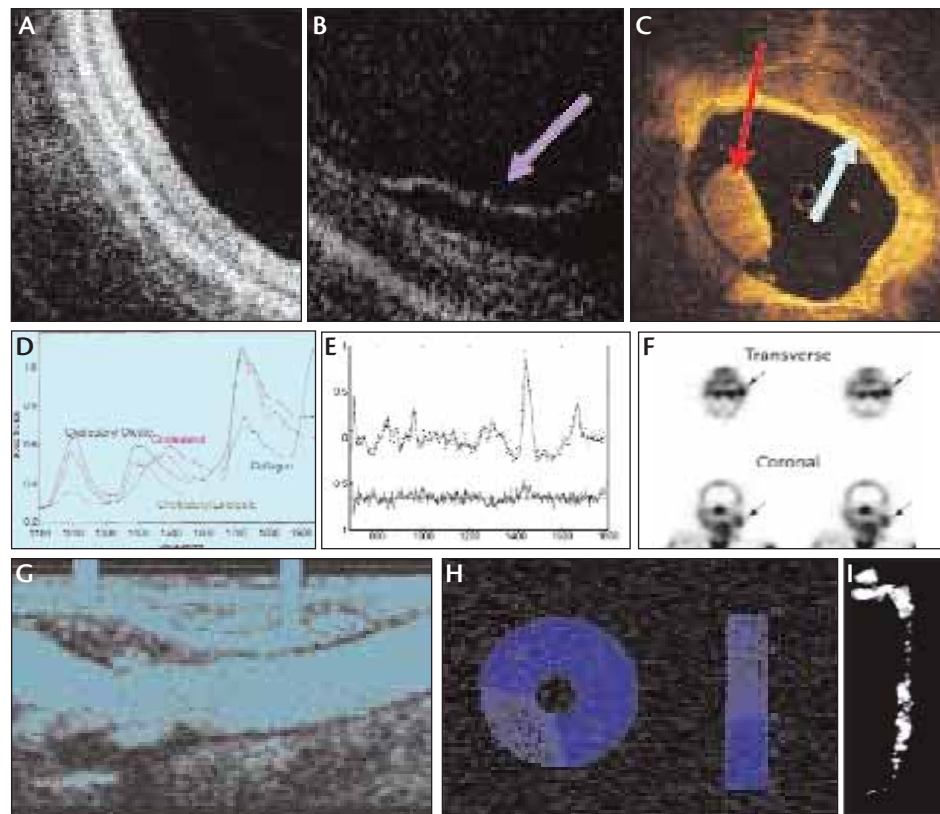
Raman spectroscopy normally uses light of a single wavelength emitted from a laser that is directed onto a tissue sample via glass fibers. The light, backscattered from the tissue, is collected and put into a spectrometer. Because nonatherosclerotic tissues have different backscattered light spectra compared to atherosclerotic tissues, composition of tissues can be differentiated. In a study conducted by Nogueira et al, 71 of the 75 postmortem human carotid artery fragments were correctly identified as nonatherosclerotic, atherosclerotic plaque without calcification, or atherosclerotic plaque with calcification when compared to histopathology.<sup>13</sup> These results are comparable to the *in vitro* human coronary arteries reported by this same group in 2002.<sup>14</sup> Using Raman spectroscopy, 95 of the 111 studied coronary artery fragments were correctly classified. Because only a small fraction of photons contribute to the Raman spectroscopy shift and an even smaller component is detected, several limitations of this technique are recognized, including poor depth discrimination and low signal-to-noise ratio.<sup>15</sup> The technique also requires substantial

postsignal processing, raising additional concerns about the reproducibility and accuracy of the technique.<sup>16</sup> Finally, Raman spectroscopy does not directly provide structural or morphological information about the lesion and must be paired with either intravascular or optical coherence tomography (OCT).

NIR reflection spectroscopy uses light of wavelengths from 800 nm to 2,500 nm. Infrared light enters arterial tissue, is scattered, possibly absorbed, and backscattered into a collection aperture and then into an optical spectrometer. In a review article by Caplan et al, seven studies were reported that documented that NIR reflection spectroscopy can accurately identify important features of atherosclerotic plaques suspected to represent plaque vulnerability; unfortunately, these features only relate to the different percentage of plaque components, including lipid, calcium, fibrous tissue, and phospholipids. Compared to Raman spectroscopy, the advantage of NIR reflection spectroscopy is deeper penetration into the atherosclerotic plaque. Unfortunately, NIR reflection spectroscopy has some of the same limitations as Raman and does not provide structural or morphological information about the lesion.

## ANNEXIN V

Apoptosis has been described in advanced human atherosclerosis and can contribute to plaque instability.<sup>17</sup> Apoptosis of smooth muscle cells results in thinning of the fibrous cap, and apoptosis of macrophages may add to the volume of the lipid core. During apoptosis, alterations in the cell membrane result in loss of phospholipid



**Figure 1.** Competing technologies to perform imaging of vulnerable plaque. OCT images demonstrate the intima, media, and adventitia of an *in vivo* porcine iliac artery (A), as well as intimal dissection (B, arrow). OCT image from a patient demonstrating luminal clot (red arrow) and lipid core with fibrous caps (C, blue arrow). NIR spectra of various plaque components (D).<sup>12</sup> Raman spectroscopy spectrum dominated by cholesterol and lipid core (E).<sup>13-16</sup> Transverse and coronal views obtained by SPECT in a patient with a stroke 3 days before imaging with uptake of radiolabeled annexin (F, arrows).<sup>17-20</sup> Enhanced vessel lumen (large arrow) and plaque (small arrow) visualization with the use of targeted ultrasound contrast agents (G).<sup>21-23</sup> Intravascular MRI image in a patient, where yellow is lipid and blue is fibrous tissue (H).<sup>24-28</sup> Near-infrared fluorescent imaging (NIRF) image of apolipoprotein E  $-/-$  mouse aorta showing cathepsin B-activated fluorescent areas in the arch and abdominal aorta (I).<sup>29-34</sup>

symmetry and exposure of phosphatidylserine on the cell surface. Annexin V, a naturally occurring protein, has a high affinity for binding to phosphatidylserine and can be used for the noninvasive detection of apoptosis.

Annexin V is a member of the annexin family of more than 160 proteins that share the property of  $\text{Ca}^{2+}$ -dependent binding to negatively charged phospholipid surfaces. For *in vivo* imaging of vulnerable plaque with single photon emission computed tomography (SPECT), annexin V can be radiolabeled with technetium, iodine, indium, or fluorine. Currently, the most studied radionuclide-labeled annexin V is technetium.<sup>18</sup>

Several disadvantages are recognized when imaging with this radioactive molecule. Technetium requires a

more complex conjugation principle to attach the atom to bioactive molecules. Replacing a hydrogen atom with a technetium ion is not possible, and it cannot serve as a substitute for carbon, nitrogen, or oxygen atoms. Furthermore, high uptake of technetium-labeled annexin V occurs in the kidneys and liver resulting in high background noise in SPECT images.

In a study conducted by Kietselaer et al, technetium-99m-labeled annexin V was detected in unstable carotid plaques in patients undergoing carotid endarterectomy.<sup>19</sup> Two patients with recent history and two patients with remote history of a transient ischemic attack (TIA) were included in the study. With SPECT imaging, annexin V uptake was observed in patients with TIAs days before imaging, while no annexin V uptake was seen in patients with TIAs months before imaging. Histopathologic characterization of the endarterectomy specimens revealed morphological features of unstable plaque, such as macrophage infiltration in patients with recent TIA.

Technetium-99m-labeled annexin V is limited by its spatial resolution, which is similar to other commonly used nuclear techniques.<sup>20</sup> Furthermore, this approach is limited to identifying apoptosis only and not the many additional features of vulnerable plaque. Also, apoptosis has only been reported to identify ruptured plaque in patients in a single study to date; that study was after plaque rupture.

## ULTRASOUND WITH MICROBUBBLES

Contrast-enhanced ultrasound techniques for imaging molecular or cellular processes have been recently achieved using site-targeted microbubbles. The gas-filled microbubbles are composed of albumin or lipid shells. The microbubbles have a diameter of 2  $\mu\text{m}$  to 5  $\mu\text{m}$  and move through the microcirculation because they are smaller than red blood cells. The microbubbles are used to enhance ultrasound images because they represent a high acoustic impedance mismatch and strongly reflect ultrasound waves.

Several strategies have been reported to target ultrasound microbubble contrast agents to lesions. One approach can identify ruptured plaques using antibodies against tissue factor. However, because tissue factor is protected by the overlying fibrous cap, the technique is not suitable to image and detect plaques at risk of rupture. A second strategy takes advantage of chemical-binding affinity of the microbubble shell to the lesion. For example, in atherosclerotic disease, an over-expression of leukocyte adhesion molecules (LAMs) in the diseased endothelium exists, and these molecules are known to have non-specific interaction with lipid shells of the microbubbles. Increased affinity between the lipid and LAMs results in

reduced transit speed of the microbubbles passing along the dysfunctional endothelium.<sup>21</sup> A third more selective strategy relies on the attachment of antibodies or other ligands to the microbubble surface that recognize disease-related antigens.<sup>22</sup> The microbubbles can be selectively targeted to P-selectin, intercellular adhesion molecule-1 (ICAM 1), and vascular adhesion molecule (VCAM), which are expressed on the lesion surface or within plaque microvessels. Unfortunately, microbubble recognition of LAMs and ICAMs are nonspecific approaches, which can identify inflammation but not unstable plaques at risk of rupture.

Currently, interrogating the entire coronary tree using contrast-enhanced ultrasound techniques is not feasible due to the tortuosity of coronary arteries. Orienting the ultrasound probe perpendicular to all coronary, cerebral, or peripheral blood vessels as they bend away from the imaging probe is not practical. Accordingly, reported results have been restricted to straight blood vessels, such as the carotid artery.<sup>23</sup> Another challenge is that the signal-to-noise ratio with existing contrast agents is low. The low signal-to-noise ratio could be caused by a number of factors, including a short life span of the microbubbles after binding, a relatively low binding affinity of the microbubbles, or a high unbinding rate. Better ligands need to be identified that have greater binding affinity, chemistry that permits stable conjugation to the microbubble surface. Finally, concerns have been voiced regarding the immunogenic potential of the antibodies associated with the microbubble. The injection of immunogenic antibodies into large segments of the patient population to screen for vulnerable plaque increases risk of large-scale systemic immunologic reactions.

## MAGNETIC RESONANCE MOLECULAR IMAGING

Magnetic resonance imaging (MRI) is emerging as an advantageous modality given its ability to demonstrate both anatomic and physiological information without radiation exposure. This technique is dependent on imaging contrast agents concentrated within a pathological site by either passive or active mechanisms.

Passive contrast agents are primarily taken up by phagocytic cell such as macrophages. These agents are metallic particles, such as iron oxide nanoparticles (50 nm to 500 nm), gold shell nanoparticles (120 nm), or carbon nanotubes (4 nm X 10 nm).<sup>24</sup> Iron oxide nanoparticles can be coated with dextran, phospholipids, or other compounds to enhance uptake. The iron produces strong local disruptions in the magnetic field of MRI scanners, which lead to increased T2 relaxation causing a decrease in image intensity in regions with iron particle accumulation.

Active contrast agents refer to ligand-directed site-specific accumulation of a contrast agent. A wide variety of ligands, including antibodies, peptides, and polysaccharides, can be used to bind specifically to cellular biomarkers.<sup>25</sup> These ligands may be attached directly or indirectly to the contrast agent. One of the drawbacks of this technique is that these ligands are recognized and cleared by the reticuloendothelial system. Therefore, surface chemistry modification such as incorporation of polyethylene glycol is often necessary to delay or avoid rapid systemic removal of the agents.

One target molecule has been fibrin, because its deposition is an early marker for plaque rupture or erosion.<sup>26</sup> Ligand composed of an antibody fragment specific for fibrin peptide domains can be used to identify the unstable plaque. Perfluorocarbon particles loaded with 50 to 90,000 gadolinium atoms per particle bind specifically to the fibrin and yield a substantial amplification of signal from fibrin clots. With this technique, the detection of disrupted plaque was demonstrated in human carotid endarterectomy specimens obtained from symptomatic patients with TIAs and strokes.

Another biomarker that can be used to localize in active atherosclerotic lesions for diagnosis and therapy is  $\alpha v \beta 3$ -integrin.<sup>27,28</sup> This molecule is widely expressed in regions of angiogenesis. Angiogenic vessels proliferate from the vasa vasorum to meet the high metabolic demands of plaque growth, and thus are employed as a marker for MRI to detect unstable plaque. Moreover, recent reports indicate that intravenous delivery of fumagillin-loaded nanoparticles (an antiangiogenic agent) targeted to  $\alpha v \beta 3$ -integrin epitopes on vasa vasorum in growing plaques resulted in therapeutic inhibition of plaque angiogenesis in cholesterol-fed rabbits. Despite these advances, MRI for patients has a spatial resolution worse than 100  $\mu m$  and hence cannot detect thin fibrous caps or individual cells.

## NIRF

NIRF consists of an excitation light source, illumination and detection optics, and a charge-coupled device for recording the fluorescent emission. The excitation light source can be either a laser or an incoherent light source (eg, arc lamp). Generally, laser sources are preferable because they offer a higher spectral radiant exitance than incoherent sources. Source light enters the tissue through an optical system (fiber or array of fibers) and can encounter a molecular probe that causes fluorescent emission in the near infrared (700 nm to 1,000 nm) that is collected and detected. In biological tissues, NIR light has a lower scattering cross-section than visible light, thus providing deeper tissue penetration.<sup>29</sup>

The molecular contrast agents fall into three main categories: nonspecific agents, targeted agents, and smart probes. Because nonspecific agents are small and can diffuse in both normal and abnormal tissues, disadvantages include poor spatial resolution due to reduced target-background ratios.<sup>30</sup> To overcome reduced enhancement, targeted approaches have been developed to increase the localization of image contrast-enhancing molecules in the diseased tissues and to reduce their uptake in the normal tissues. However, this technique depends largely on the receptor density of interest and the specificity of the antibody being used. Furthermore, differentiating bound from unbound probes is difficult. Unlike nonspecific or targeted agents that always fluoresce, smart probes can change their physical properties in response to a specific molecular interaction. These probes are not light-emitting in their native state and fluoresce only upon interaction with specific proteases, such as cathepsins and MMPs, which are produced by macrophages and overexpressed in human atherosclerosis.<sup>31,32</sup> For instance, the chemical bond maintaining the fluorescent probes in close proximity in a quenched state, when hydrolyzed by cathepsins and/or MMPs, allows the probes to move apart and then interact with NIR excitation light.

Organic and inorganic contrast agents have been used as fluorescent molecular probes. One important class of organic fluorophores is the heptamethine cyanines, although this organic fluorophore has limitations.<sup>33</sup> First and foremost, controlling excitation and emission wavelengths is difficult. Tuning a fluorophore to precise excitation emission wavelengths requires sophisticated chemistry. Second, these contrast agents are not biologically compatible. Without charged groups, heptamethine indocyanines can be toxic due to intracellular accumulation.<sup>34</sup> Although the disulfonated indocyanine has been used routinely in humans for more than 40 years and has an excellent safety profile (green dye used to determine cardiac output), other heptamethine indocyanine proposed by groups identifying cathepsins and MMPs are not FDA approved for human use. Finally, although these probes offer diagnostic opportunities, therapeutic potential for vulnerable plaque appears low.

## OCT

Since OCT was introduced in the early 1990s, many investigators have recognized the potential application to image vulnerable plaques. Recently, Tearney et al have used OCT to identify macrophages in atherosclerotic plaque.<sup>35</sup> They correlated greater intraplaque concentration of postmortem macrophages by CD68 immunohistochemical staining with regions of increased OCT light

reflection. The same investigators have extended these studies to identify macrophages in coronary arteries of patients using intravascular OCT.<sup>36</sup> However, limitations in the specificity of this approach to identify macrophages are noted; for instance, although dark regions in OCT images correlated with macrophage presence in histology, contributions from other tissue components could not be ruled out. Use of conventional OCT to identify macrophages with high confidence in plaque lesions and thereby evaluate macrophage density *in vivo* is problematic due to confounding contributions from other tissue components. Need for approaches with greater specificity for macrophage imaging are recognized.

Magnetomotive OCT was recently demonstrated to enhance image contrast using magnetic particles *in vivo* in tadpoles by measuring optical scattering changes.<sup>37</sup> However, the approach by Boppart et al did not provide a quantitative phase-based measurement of tissue displacement. In contrast, Oh et al reported cellular imaging of tissue-based macrophages and distinguished them from other plaque components by tagging only macrophages with iron nanoparticles.<sup>38,39</sup>

OCT is a rapidly maturing imaging technology to identify additional features of vulnerable plaque including thin fibrous caps, large lipid cores, and plaque composition. Linking these high-risk anatomic features with the specific identification of macrophages as an additional high-risk feature appears feasible. All OCT studies performed to date have determined plaque composition on human postmortem specimens whose optical properties are different from living tissue. Birefringence of elastin and collagen in the fibrous cap can decrease rapidly after tissue removal from the living organism. Brezinski and Fujimoto were the first to demonstrate that fibrous cap thickness and lipid pool size on postmortem specimens could be imaged with OCT.<sup>40,41</sup> They further demonstrated that the presence of calcium did not interfere with either the penetration or reflection of light from these plaques. Because human plaques calcify with age, the ability of OCT to eliminate calcium as a source of artifact when ultrasound and CT-based approaches cannot is an important strength of our approach. However, these studies were not performed in a statistically rigorous fashion. Bouma and Tearney subsequently performed a more detailed study and demonstrated that postmortem plaque could be categorized into those composed of primarily fibrous, lipid, or calcium with high sensitivity and specificity when compared to the gold standard of histology.<sup>42,43</sup> Feldman and Milner were the first to demonstrate that features of vulnerable plaques (thin fibrous caps and

large lipid cores) in live apolipoprotein E knockout mice could be identified.<sup>44</sup>

One constraint for OCT imaging of atherosclerotic plaques within the arterial lumen is strong scattering of light by red blood cells. Once a catheter system is positioned in an artery, scattering by blood between the OCT probe and artery strongly attenuates light penetration into the vessel wall. One proposed solution is use of saline flushes and is robust when coupled with Fourier-domain OCT. Fourier-domain OCT increases imaging speeds by more than 100-fold compared to the previous time-domain approaches.<sup>45</sup> Fourier-domain OCT has been demonstrated to allow imaging a 40-mm arterial segment using a single 10 mL saline flush.<sup>46</sup>

When repeated or longer pullback times are required, a proposed solution is use of artificial hemoglobin as a substitute for saline.<sup>47</sup> Artificial hemoglobin is nonparticulate and therefore does not scatter light. Moreover, artificial hemoglobin is about to be approved by the US FDA as a blood substitute and can carry oxygen, preventing myocardial ischemia that can occur from prolonged saline flushes.

## CONCLUSION

To reduce the risk of MI and CVA, vulnerable plaques will need to be identified in a prospective fashion. Currently, many competing modalities are under study to identify the features that predict plaque rupture. The authors think that OCT may offer the most robust potential to identify these features. For instance, Raman spectroscopy and NIR spectroscopy have to be coupled with either intravascular ultrasound or OCT because they cannot provide structural or morphological information about the plaque. Using technetium-99m-labeled annexin V to identify apoptosis is a novel concept, but, similar to other commonly used nuclear imaging techniques, this modality is limited by its spatial resolution. Ultrasound with microbubbles and MRI have limited spatial resolution (less than 100  $\mu\text{m}$ ) and hence cannot detect thin fibrous caps or individual cells. NIRF imaging of vulnerable plaque is interesting but offers no therapeutic options.

OCT has the highest resolution (<10  $\mu\text{m}$ ) of all these technologies, and thus has great potential for imaging vulnerable plaques. In addition to demonstrating features of vulnerable plaques (thin fibrous caps and large lipid cores), OCT can also identify macrophages with high confidence in plaques. With the advancement of Fourier-domain OCT, 40-mm arterial segment can be interrogated using a single 10 mL saline flush, and the epicardial vessel can be imaged in the future without the need for balloon occlusion. Finally, optical technology

utilized in OCT systems is expected to advance rapidly, and OCT images of the cardiovascular lumen will have greater contrast, higher signal-to-noise ratios, and increased specificity of constituents. Inasmuch as light offers an extraordinary signal bandwidth and has the capacity to carry a tremendous volume of representative information about a tissue, capability of OCT for cardiovascular imaging has not been fully realized. Specifically, the polarization and phase sensitivity of light provide unique imaging capabilities and tissue characterization that are in the early stages of development. As OCT technology advances, the potential to reduce risk of MI and CVA will increase; the technology has excellent potential to contribute to improved cardiovascular health. ■

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