

# Molecular Imaging of Myocardial and Vascular Disorders With Ultrasound

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Methods for noninvasive imaging of specific disease-related molecular changes are being developed in order to expand and improve diagnostic capabilities and to enhance therapeutic decision making in the clinical setting. These new techniques have also started to be incorporated into research programs in order to better characterize pathophysiology or evaluate treatment efficacy. Molecular imaging with contrast-enhanced ultrasound relies on the detection of the acoustic signal produced by either microbubbles or other acoustically active particulate agents that are targeted to sites of disease. This review describes the progress that has been made in the development and testing of methods for contrast ultrasound molecular imaging of cardiovascular disease. Specifically, topics that will be addressed include: 1) the bioengineering and detection schemes for targeted probes; 2) specific disease processes (myocardial ischemia, atherosclerosis, and transplant rejection) where molecular imaging may play a role; and 3) the potential role of ultrasound as a molecular imaging technique. (J Am Coll Cardiol Img 2010;3:204–11) © 2010 by the American College of Cardiology Foundation

## Overview of Molecular Imaging

The term *molecular imaging* has been used to describe a broad family of imaging techniques used in the basic science and clinical settings. For the clinician, the term refers to methods that have been developed to evaluate pathophysiologic changes at the molecular level in vivo with medical imaging techniques and usually the administration of a targeted contrast agent that binds to or is activated by a pathologic molecule of interest. It is likely that this technology will further enhance the already indispensable role noninvasive imaging already plays in the management of patients with cardiovascular disease by improving diagnostic performance or allowing early detection of disease. Profiling disease phenotype could also be used to select the most appropriate treatment therapy based on the specific pathway activation. This notion is

probably more familiar to those in cancer medicine where chemotherapeutic or antiangiogenic regimens are starting to be matched to the molecular patterns on imaging or pathology. It should also be mentioned that molecular imaging techniques could be used to evaluate new therapies and whether they justify further development in the pre-clinical or clinical stage.

The translation of molecular imaging technology to patients relies on matching the targeted probes to the clinical purpose. For example, early detection of atherosclerosis will require targeting of the sentinel events of atherosclerosis such as the presence of oxidized lipoproteins in the vessel wall or endothelial cell activation; whereas risk assessment in those with more established disease will likely require probes that can evaluate macrophage content, protease activity, apoptosis, or prothrombotic milieu. It is, therefore, not surprising that a

myriad of different targets involved in atherosclerosis, ischemia, and angiogenesis have been explored in pre-clinical studies. It should also be mentioned that the imaging technique must also be matched to clinical purpose in terms of sensitivity, spatial resolution, and practical issues of cost and ease of use.

This review will focus on one form of molecular imaging, targeted contrast-enhanced ultrasound (CEU). This technique relies on the selective targeting and retention of microbubbles or acoustically active nanoparticles at sites of disease. These agents are generally confined to the vascular space except in situations of extraordinary vascular injury. For evaluation of myocardial and vascular diseases, targeted ultrasound probes have generally been used to evaluate endothelial phenotype or activation and adhesion of immune cells. Contrast agents that are targeted to thrombus or for use in gene/drug delivery have also been developed but will not be covered in this review.

### Bioengineering of Targeted Ultrasound Contrast Probes

Microbubble and submicrometer ultrasound contrast agents generally have a stabilizing polymer or albumin “shell” or a surfactant layer in the case of lipid-stabilized agents. Encapsulation enhances *in vivo* stability of microbubble contrast agents by reducing surface tension and preventing outward diffusion of the gas core component (1). For the purposes of molecular targeting, the shell also serves as the interface between the gas bubble and the biologic microenvironment.

A simple strategy for microbubble targeting has been to seize upon the ability of the innate immune system to recognize and remove foreign particles. Microbubbles with either lipid or albumin surfaces can activate serum complement locally at the bubble–blood interface (2–4). Activated complement components such as C3b on the microbubble surface mediate interactions between microbubbles and activated leukocytes. These interactions have been used to image acute and severe cellular inflammatory responses in acute myocardial infarction, severe chronic ischemic left ventricular dysfunction, and transplant rejection (Fig. 1) (5–7). Complement can also mediate interactions between endothelial cells and microbubbles with either albumin or lipid shell (4,8,9). This interaction provides a simple method for detecting chronic or acute endothelial activation in animal models of atherosclerosis and vascular injury (4,9).

For lipid microbubbles, the sensitivity for detecting leukocyte activation and cellular adhesion can be increased by the addition of lipids that carry a strong net negative charge thereby amplifying complement activation, or by eliminating the polyethyleneglycol surfactant layer that creates a steric boundary (8).

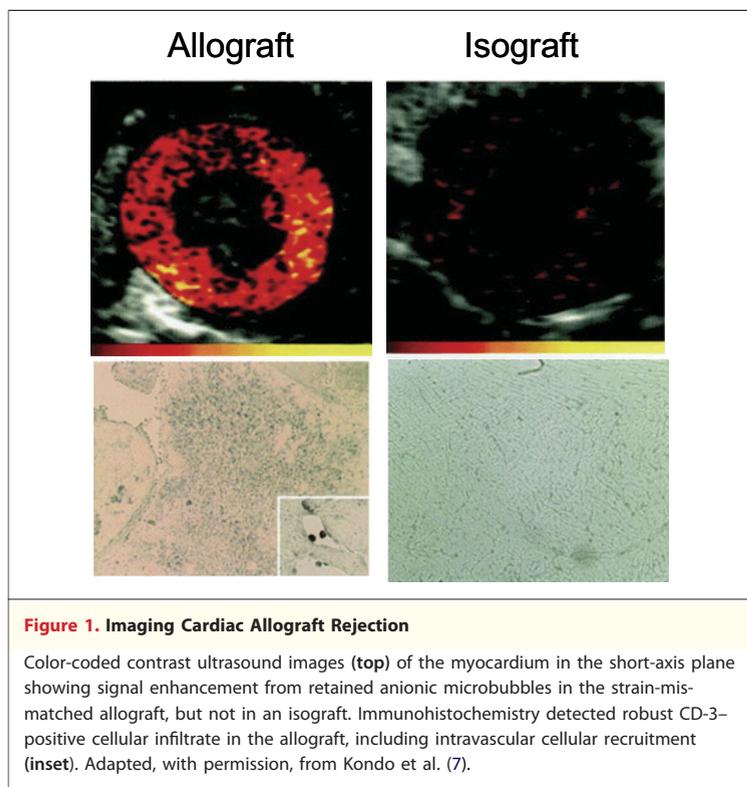
A more specific and more efficient targeting approach has been to conjugate ligands against disease-related molecules to the surface of contrast agents. Examples of the type of ligands include monoclonal antibodies (mAb), peptides, glycoproteins, and peptidomimetic small molecules. The most common strategy has been to conjugate these ligands to a bifunctional molecule that has a lipid membrane anchor domain (e.g., hydrophobic polycarbon tail) and also a molecular spacer that projects the ligand away from the shell surface.

The attachment of multivalent micro- or nanoscale contrast agents to disease molecules depends on host-related factors (site, density, and location of the target molecule; presence of endogenous inhibitors; shear forces) as well as bubble-related factors. For the latter, the surface density of ligands is an important issue that in flow chamber experiments tends to affect binding efficiency at medium to high shear rates (Fig. 2A) (10). Based on the data that have been generated for lipid microbubbles, most targeted imaging protocols *in vivo* are performed with agents that contain several thousand ligands per square micrometer of shell surface area. Similarly, the site density of the target molecule tends to affect binding capacity of microbubbles (Fig. 2B) (11).

Ideally, the kinetic properties of the ligand that is used to target ultrasound contrast agents needs to be matched to the intended microenvironment. For example, ligands with a rapid on-rate, such as those from catch bonds, may be advantageous for initiating interaction at high shear, whereas in certain circumstances firm attachment may be best achieved through low “off-rate” bonds such as those mediated by mAb. Hence, bioengineering of microbubbles must be performed with the vascular conditions in mind. Recent data have suggested that a dual ligand approach could be advantageous for high shear binding where, similar to leukocytes and platelets, a fast on-rate ligand allows initial interaction with the vascular interface with subsequent formation of bonds with a low off-rate ligand (12).

#### ABBREVIATIONS AND ACRONYMS

<b>CEU</b>	= contrast-enhanced ultrasound
<b>ECAM</b>	= endothelial cell adhesion molecule
<b>ICAM-1</b>	= intercellular adhesion molecule-1
<b>mAb</b>	= monoclonal antibody
<b>VCAM-1</b>	= vascular cell adhesion molecule-1



### Detection of Infarction and Acute Ischemia

The diagnosis of acute coronary syndromes currently relies on combining clinical information with widely available diagnostic tests such as electrocardiogram and serologic markers of myocellular necrosis such as troponins. Because of the well-recognized limitations in the diagnostic accuracy of this approach (13,14), newer methods for rapid diagnosis of ischemia and/or infarction are being developed. Targeted imaging of the molecular signature of ischemic injury could be used not only for diagnosis, but also to map the spatial extent of involvement, which can be important for guiding therapy or assessing prognosis. Advantages of an ultrasound approach include the ability to perform bedside testing and the speed of the imaging protocols.

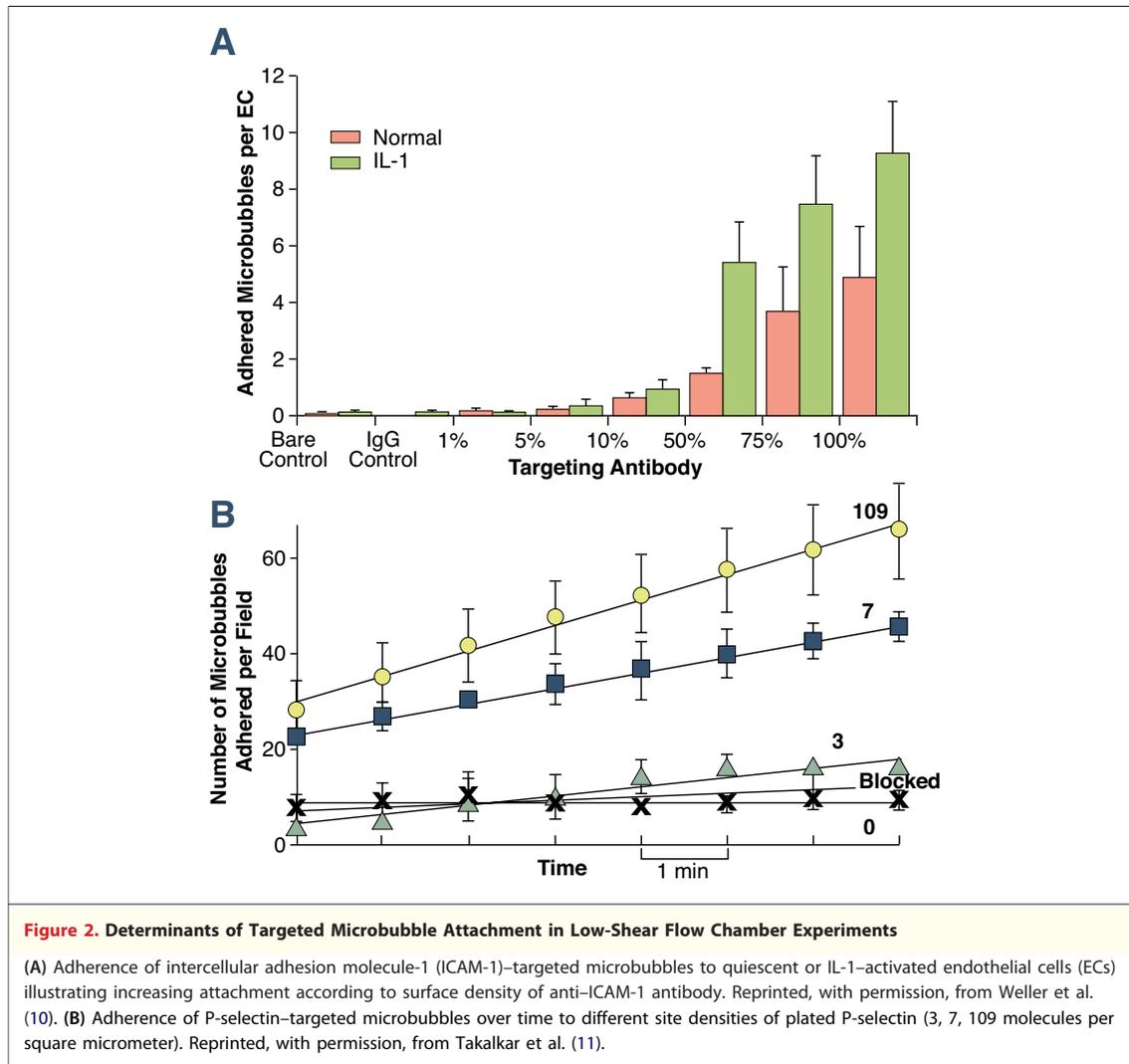
Complement-mediated microbubble-leukocyte interactions described earlier have been used to detect ischemic injury severe enough to produce infarction (4). There are several caveats to this approach. First, the region of enhancement with leukocyte-targeted microbubbles in myocardial infarction is time-dependant because of the dynamic spatial pattern of immune cell recruitment. Hence, the distribution of leukocyte-targeted signal tends to encompass the entire risk area early ( $\leq 90$  min)

after reperfused myocardial infarction, but then gradually contracts to the region of infarction (4). A second consideration is that leukocyte targeting is unreliable in regions of microvascular no-reflow where microbubble flux is absent.

The sensitivity of the leukocyte-targeted imaging approach has been insufficient for diagnosing ischemia that is mild and does not produce infarction. This situation has required the creation of microbubble probes capable of detecting modest endothelial activation. For this purpose, microbubbles have been targeted to endothelial selectins (P-selectin, E-selectin). P-selectin is stored pre-formed in endothelial cell, is externalized within minutes of even mild injury or ischemia, and can persist for hours after ischemic insult (15,16). Hence, it is an ideal target for detecting myocardial ischemia that is ongoing or has resolved. P-selectin-targeted imaging was first described in renal ischemia-reperfusion injury (17). It has since been used to detect and spatially define recent myocardial ischemia in mice and rats (18,19). Targeting has been achieved through the use of mAb, or glycoprotein or carbohydrate mimetics of the endogenous P-selectin glycoprotein ligand-1 (PSGL-1). The nonantibody targeting techniques have the theoretic advantage of binding to E-selectin as well, which could extend the window in which post-ischemic injury can be detected.

### Early Detection of Atherosclerosis

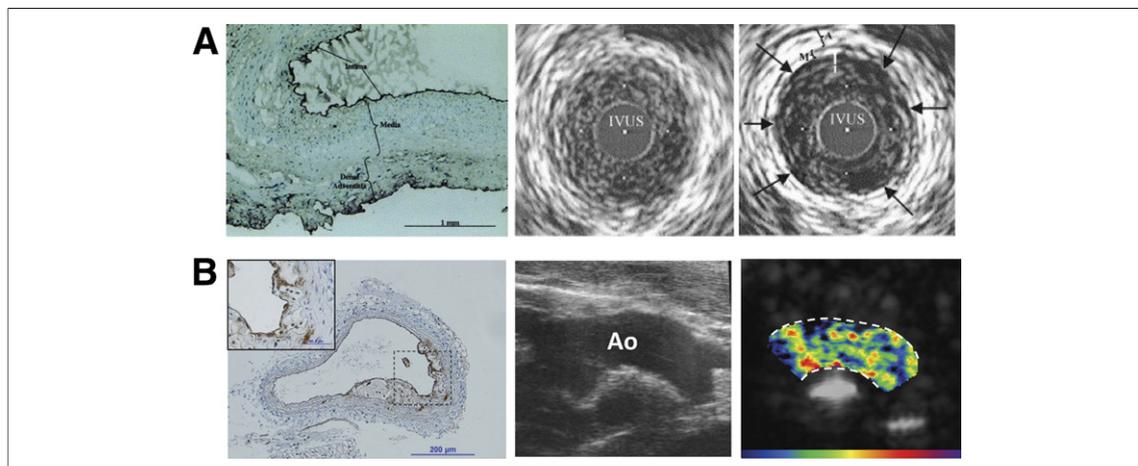
It is now widely accepted that atherosclerotic lesion severity in terms of luminal encroachment or plaque volume (taking into account eccentric remodeling) does not provide a complete assessment of risk. Plaque composition is a key feature for predicting adverse events (20). Molecular imaging can provide insight and prognostic information on the biologic processes that lead to unstable plaque growth or acute atherothrombotic events. Most ultrasound contrast agents do not have access to high-risk cellular features or molecular profile of the intraplaque environment unless there is drastic alteration of local vascular permeability (21). Hence, CEU molecular imaging of atherosclerosis has focused on detection of endothelial cell activation on the plaque surface or in the underlying vasa vasorum; or detection of a prothrombotic environment. Endothelial cell adhesion molecules (ECAMs) such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), or selectins regulate entry of immune cells that are



critical for the initiation of atherosclerosis and are a major determinant of plaque instability (22–24). Targeted imaging of ICAM-1 and VCAM-1 in miniswine models of atheroma produced by vascular mechanical injury and cholesterol-rich diet has been achieved with intra-arterial injection of immunoliposomes that are rendered echogenic by manufacturing processes that promote air entrapment (Fig. 3A) (25). More recently, different degrees of plaque inflammatory burden produced by dietary manipulation in apolipoprotein-E<sup>-/-</sup> mice had been discerned by CEU detection of endothelial VCAM-1 up-regulation after intravenous administration of targeted microbubbles (Fig. 3B) (26). There is minimal vasa vasorum component of disease in this murine model, indicating that endothelial attachment of targeted microbubbles is possible even in the face of very high shear stress of the murine aorta. Flow chamber studies suggested that

this was possible because of the pulsatile nature of flow in large vessels and the ability for agent to adhere during low-shear diastolic periods (26). Recent studies have demonstrated that molecular imaging of endothelial activation with VCAM-1 and P-selectin-targeted microbubbles can detect very early atherosclerotic changes even before there is lumen encroachment (27).

Targets other than ECAMs could be used to evaluate high-risk plaque phenotype with CEU. Echogenic perfluorocarbon nanoparticles targeted to tissue factor have been shown to enhance the pig carotid arteries after overstretch injury (21). It is not currently known whether plaque permeability for this agent is sufficient for a tissue factor targeting approach in the absence of physical trauma. Complement-mediated attachment of albumin microbubbles to the vascular endothelium has been used to detect local activation of the innate immune



**Figure 3. Molecular Imaging of Atherosclerosis**

(A) Immunohistochemistry demonstrating ICAM-1 expression in the femoral artery of a cholesterol-fed Yucatan miniswine several weeks after balloon injury, and intravascular ultrasound (IVUS) after arterial injection of control (middle panel) and ICAM-1-targeted (right panel) immunoliposomes. Vessel wall enhancement (arrows) was seen only for the ICAM-1-targeted probe. Reprinted, with permission, from Hamilton et al. (25). (B) Immunohistochemistry demonstrating VCAM-1 expression in an apolipoprotein E<sup>-/-</sup> mouse on high-fat diet, high-frequency (40 MHz) ultrasound of the aortic arch in long axis demonstrating mural thickening (middle panel), and molecular imaging (right panel) 10 min after intravenous injection of VCAM-1-targeted microbubbles (color scale at bottom). Ao = aorta. Reprinted, with permission, from Kaufmann et al. (26).

system associated with vascular injury or severe atherosclerosis (4,8). There is also promise that targeted microbubbles may be able to detect proliferation of the vasa vasorum and plaque neovascularization with agents targeted to adhesion molecules or to endothelial epitopes with relative specificity angiogenic vessels. However, this phenomenon has not yet been definitively shown.

### Heart Transplant Rejection

Conventional methods for detecting cardiac allograft rejection with noninvasive imaging are based on myocardial function. This approach is somewhat insensitive for detecting rejection in the early stages or in those with pre-existing dysfunction. There have been few advances in echocardiographic screening for rejection apart from using more sensitive strain- or tissue velocity-based methods for evaluating function. The ability to target the cellular immune response during heart transplant rejection is a promising application for CEU molecular imaging. Complement-mediated interactions between activated leukocytes and anionic microbubbles have been shown to detect heterotopic heart transplant in a rat model of strain mismatch and to assess the response to immunomodulatory therapy (7). In a similar rat model, microbubbles targeted to

ICAM-1 have been used to detect endothelial activation in acute rejection of cardiac heterografts (21). Implicit in these results is that CEU molecular imaging could also be useful for evaluating patients with suspected acute myocarditis.

### Imaging Vascular Adaptation to Ischemia

CEU molecular imaging has been used to detect some of the key biologic processes involved in vasculogenesis and arteriogenesis. It is difficult to argue that there is currently a clinical demand for “angiogenesis imaging” in ischemic heart or limb disease. However, the pairing of CEU molecular imaging and perfusion imaging has been used as a tool to study vascular adaptation to chronic ischemia and to evaluate potential treatment strategies. It may also be useful in atherosclerotic disease for detecting vasa vasorum remodeling which is an important determinant of inflammatory burden and risk (28).

Molecular imaging of vascular remodeling in ischemic limb models has been performed with microbubbles targeted against endothelial integrins (alpha<sub>v</sub>-integrins) that are thought to be involved in migration, adhesion, and survival signaling (29). In these studies, signal enhancement from microbubbles bearing the alpha<sub>v</sub>-integrin ligand echistatin tended to herald changes in flow, particularly with growth factor stimulated arteriogenesis. Vas-

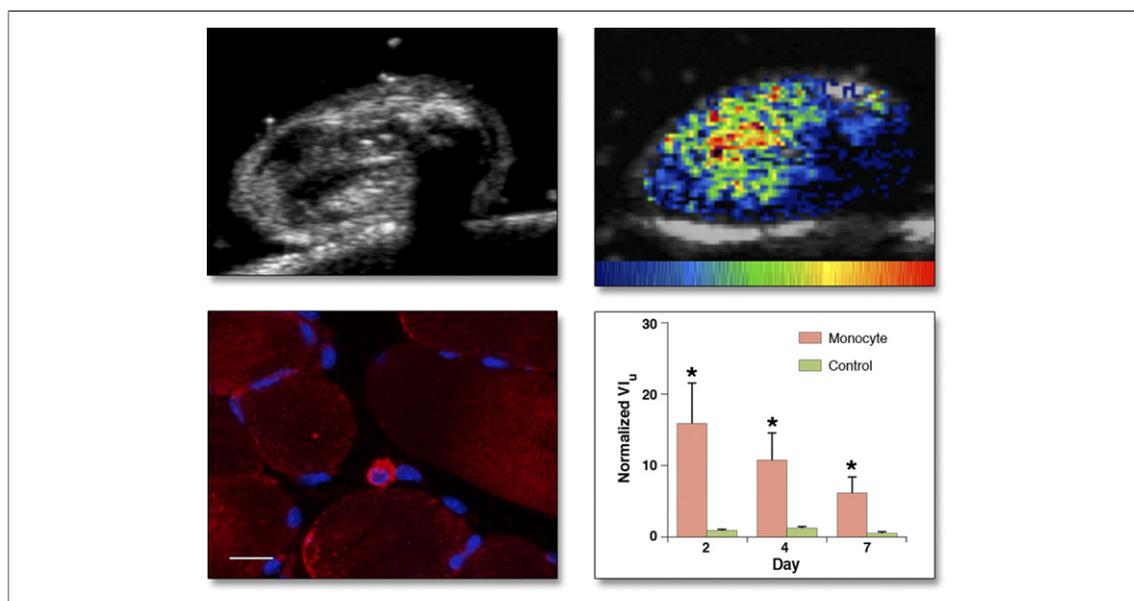
cular remodeling can also be detected by targeting microbubbles directly to growth factor receptors, such as VEGFR-2 expressed during vasculogenesis and angiogenesis, although experience has been limited mostly to tumor rather than ischemic models (30). CEU molecular imaging of leukocyte activation, monocyte recruitment, and up-regulation of ECAMs has been used to characterize different components of the immune response that are requisite in vasculogenesis and ischemia-mediated angiogenesis (Fig. 4) (31). Evaluating the inflammatory response in angiogenesis may provide an important tool for understanding and optimizing proangiogenic cytokine/chemokine therapy that may have an immunomodulatory mode of action (32).

#### Advantages and Limitations to the Technique

There are several relative advantages of using a contrast ultrasound-based approach for molecular imaging in both the clinical and research settings. Some of the most pertinent advantages are practical issues such as cost, speed, and ease of use. The technique does not require any specialized imaging equipment, and the protocols can be completed in only 8 to 10 min. The short duration of the protocols, short circulating half-life, and the ability

to destroy the contrast agent after image acquisition provides the opportunity to perform sequential injections. This is a key issue that allows one to temporally characterize expression profile and to image several different molecular targets during a single relatively brief imaging period. The ability to almost simultaneously acquire structural and/or functional information together with molecular signal is also an advantage of an ultrasound-based approach.

There are also important limitations for CEU molecular imaging. First and foremost is the inability to target events that occur outside of the vascular compartment. Hence, it is unable to evaluate certain processes that are important in cardiovascular disease such as matrix remodeling, cellular apoptosis, and oxidized lipid accumulation. Attachment of microbubble contrast agents are shear dependent (26). This problem, however, may be solved with the use of targeting moieties that normally act through a “catch bond” (such as recombinant PSGL-1 or GP1b) and are able to produce capture at high shears. Finally, the complex particle-based nature of ultrasound and the need for chemical conjugation of surface targeting molecules will necessitate extensive safety testing by regulatory agencies before any product will be considered for clinical use.



**Figure 4. Targeted Imaging of Monocyte Recruitment in Ischemia-Mediated Arteriogenesis**

Images at the top illustrate a B-mode image and color-coded ultrasound molecular imaging for monocyte  $\alpha_5$ -integrin for the proximal hindlimb of a mouse 2 days after iliac artery ligation. Immunohistochemistry demonstrated  $\alpha_5$ -integrin-positive (red) monocyte infiltration. Quantitative data illustrate selective signal enhancement for  $\alpha_5$ -integrin-positive monocytes in the ischemic limb which persists for several days after iliac artery ligation. Adapted, with permission, from Behm et al. (31). \* $p < 0.05$  versus control.

## Summary

Many of the key biologic processes responsible for ischemic and/or inflammatory diseases of the myocardium and vascular system are being targeted by molecular imaging in the hope that unique diagnostic and research applications will follow. The role of contrast ultrasound will undoubtedly be related not only to sensitivity for detecting these processes, but also to practical matters of cost and convenience. Evidenced by the scientific achievements summarized in this review, the diagnostic applications of ultrasound molecular imaging re-

main in the "feasibility" stage where single targeted agents are tested in animal models of established disease. The transition to clinical testing will depend, not on technical factors, but rather on the recognition of situations in which molecular imaging can provide unique information that is critical for treatment decisions.

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**Key Words:** contrast ultrasound  
■ microbubbles ■ molecular  
imaging.