Contrast ultrasound molecular imaging of inflammation in cardiovascular disease

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The cellular immune response plays an important role in almost every major form of cardiovascular disease. The ability to image the key aspects of the immune response in the clinical setting could be used to improve diagnostic information, to provide important prognostic or risk information, and to customize therapy according to disease phenotype. Accordingly, targeted imaging probes for assessing inflammation have been developed for essentially all forms of medical imaging. Molecular imaging of inflammation with contrast ultrasound relies on the detection of targeted microbubble or other gas-filled particle contrast agents. These agents are confined to the vascular space and, hence, have been targeted to either activated leucocytes or endothelial cell adhesion molecules that are upregulated in inflammation and mediate leucocyte recruitment and adhesion. This review focuses on the inflammation-targeting strategies for ultrasound contrast agents and how they have been matched to cardiovascular disease states such as myocardial ischaemia, infarction, atherosclerosis, transplant rejection, and arteriogenesis.

KEYWORDS
Inflammation; Contrast ultrasound; Molecular imaging; Myocardial contrast echocardiography

A wide array of non-invasive molecular imaging techniques for evaluating tissue phenotype in vivo have been developed and tested in humans and animal models of disease. The most common strategy for molecular imaging has been to modify contrast agents in order to change their kinetic profile and target them to molecules or cells that mediate pathophysiology. The use of these techniques in both the research and clinical arena may overcome some of the shortfalls of current imaging approaches that are generally able to evaluate anatomy and/or whole organ physiology. The ability to image disease at the molecular level could potentially be used to: (1) improve our understanding of pathophysiology of disease; (2) diagnose life-threatening diseases at an early stage, (3) monitor disease progression; (4) assess response to new or established therapies, and (5) select the most appropriate of effective therapy based on disease phenotype.

For cardiovascular applications, the ability to image the immune response is an important goal. The immune response plays an important role in most major cardiovascular ailments (Table 1). Traditional imaging approaches for evaluating cardiac or vessel anatomy or function do little to detect or quantify the inflammatory component of these diseases. More recently, targeted imaging technologies have been developed to evaluate both acute and chronic immune responses in cardiovascular disease. Some of the technical characteristics that differentiate the various forms of molecular imaging that have been used to evaluate the immune response are provided in Table 2. This review will summarize contrast-enhanced ultrasound (CEU) strategies for imaging inflammatory response in cardiovascular disease. This technique relies on the selective detection of targeted microbubble or nanoparticle contrast agents. Most of these agents are confined to the intravascular compartment. Accordingly, they have been engineered to target key regulatory events that occur within the vascular space such as leucocyte adhesion or endothelial cell activation.

1. Components of the immune response relevant to imaging

The vascular endothelium provides a structural and functional interface between circulating blood cells and the interstitial space. Activation of the vascular endothelium in response to injury or inflammatory stimuli is a critical event during acute and chronic inflammation (Figure 1). Activation involves the expression of endothelial cell adhesion molecules that interact with counterligands on the leucocyte surface, some of which require cytokine-mediated activation. The initial step of leucocyte recruitment involves leucocyte capture and rolling along the
endothelial surface. In muscle tissue, this mostly occurs in post-capillary venules and is mediated by the interaction between selectins expressed by either the endothelium (P- and E-selectin) or leucocytes (L-selectin), and their constitutively expressed leucocyte or endothelial glycoprotein counterligands. Leucocyte rolling can occur within minutes of tissue injury because of a recruitable repository of P-selectin that is pre-stored in secretory granules (Weibel-Palade bodies) of endothelial cells. Slow rolling also facilitates the slow on-rate bond formation for ICAM-1 and VCAM-1. Activated leucocytes adherent to the venular endothelium may then undergo transendothelial migration according to chemokine signals.

Contrast ultrasound imaging of inflammation has been achieved by targeting contrast agents to either activated immune cells or the endothelial cell adhesion molecules that regulate leucocyte trafficking and adhesion. Selection of the most appropriate targeting strategy employed has relied on several considerations. One consideration is whether acute or chronic inflammatory processes are of interest. For example, the acute cellular immune response to injury or ischaemia involves a coordinated sequence that is dominated by neutrophils early (<12 h) with a subsequent monocyteic response. Imaging the cellular immune response in chronic disease is more likely to involve targeting of monocytes and certain lymphocyte populations. There may also be other situations where identification of a specific immune subset is desirable. For example, there may be times where it is necessary to differentiate the monocytic and lymphocytic response, or to identify the recruitment of specific monocyte populations with regard to function. Finally, one must also take into consideration the specificity of the molecule for the disease state. For example, although certain endothelial cell adhesion molecules such as ICAM-1 are upregulated in atherosclerosis, their constitutive expression may preclude their use for early detection of atherosclerotic disease. Regardless of the target, one feature of targeted microbubbles that differentiates them from diffusible contrast agents used with other imaging modalities is that signal enhancement during CEU molecular imaging reflects only processes that are occurring within the vascular compartment.

2. Non-specific cell attachment of encapsulated agents

One strategy for imaging inflammation relies on non-specific interaction of microbubbles with activated leucocytes adherent to the microvascular endothelium, thereby resulting in tissue retention. Both albumin and lipid-shelled microbubbles can bind to activated leucocytes. Attachment of albumin-shelled microbubbles is mediated at least in part by the leucocyte β2-integrin Mac-1 which is capable of binding denatured albumin. Lipid microbubbles attach almost exclusively from opsonization whereby activated serum complement on the bubble surface interacts with complement receptors expressed upon leucocyte activation. Chemical modification of lipid shell components such as the addition of phosphatidylserine (PS) increase the binding avidity of microbubbles for leucocytes and can amplify signal enhancement during the acute inflammatory response to ischaemia. This effect can explain in part by the increased complement activation by membrane lipids with a net negative charge. Introduction of protective polymer surfactants such as fatty acid–PEG moieties that create a ‘brush’ surface can reduce complement-mediated interactions of bubbles with cells.

The strategy of non-specific targeting of leucocytes is most effective when used to image severe acute inflammatory conditions where the density of activated leucocytes on the vessel surface is high. Myocardial contrast echocardiography (MCE) performed several minutes after intravenous injection of PS-containing lipid microbubbles has been used to spatially

Table 1 Cardiovascular diseases with an inflammatory component

<table>
<thead>
<tr>
<th>Disease</th>
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<tbody>
<tr>
<td>Atherosclerosis (initiation and progression)</td>
</tr>
<tr>
<td>Unstable plaque events (rupture, erosion, thrombus, neovascularization)</td>
</tr>
<tr>
<td>Acute myocardial ischaemic injury or infarction</td>
</tr>
<tr>
<td>Chronic ischaemic ventricular dysfunction</td>
</tr>
<tr>
<td>Ventricular remodelling</td>
</tr>
<tr>
<td>Adaptive angiogenesis/arteriogenesis</td>
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<td>Allograft rejection</td>
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<tr>
<td>Myocarditis</td>
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<td>Valve degeneration/stenosis</td>
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Table 2 Characteristics of methods used for molecular imaging

<table>
<thead>
<tr>
<th>Method</th>
<th>CEU</th>
<th>SPECT</th>
<th>PET</th>
<th>MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spatial resolution</td>
<td>++</td>
<td>+</td>
<td></td>
<td>++++</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Target distribution</td>
<td></td>
<td>Intra-vascular</td>
<td>Diffusible</td>
<td>Diffusible</td>
</tr>
<tr>
<td>Temporal resolution</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Affected by shear</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Unknown</td>
</tr>
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CEU, contrast enhanced ultrasound; SPECT, single photon emission computed tomography; PET, positron emission tomography; MRI, magnetic resonance imaging.
assess the cellular immune response in a canine model of reperfused acute MI (Figure 2). This technique is able to not only detect leucocyte activation within the infarct zone, but also the lower-grade and shorter-duration inflammatory cell recruitment that is known to occur in non-infarcted but ischaemic risk area, including in collateralized zones. Since signal from PS microbubbles reflects active leucocyte recruitment but not cumulative transmigration, it has potential advantages for evaluating acute responses to therapies that are aimed at attenuating the post-ischaemic inflammatory response, since extravasated leucocytes do not contribute to the CEU signal. Moreover, the rapid imaging protocols used with CEU could be of considerable benefit in this setting. One disadvantage illustrated in Figure 2 is that inflammatory status cannot be assessed in regions lacking microvascular reflow because of the absence of targeted microbubble entry into these territories.

Retention of anionic microbubbles has also been used to image heterotopic heart transplant rejection in a rat model of strain mismatch where contrast intensity correlated well with the histological grade of rejection. Similar to the situation with reperfused infarction, signal from retained microbubbles presumably reflected the active phase of leucocyte recruitment. In this study, the early response to immunomodulatory therapy could also be gauged with MCE and anionic microbubbles, thereby supporting the notion that imaging the phase of a leucocyte recruitment could be advantageous for examining acute effects of therapy. In certain situations, complement-mediated attachment of microbubbles to the vascular endothelium can occur. Microbubbles that possess a strong anionic surface charge and that lack a PEG protective surface can be retained in vessels of animal models of severe atherosclerotic disease with and without balloon injury. These interactions also are inhibited by complement depletion. It is likely that the endothelial mediator(s) of anionic and lipid microbubble attachment are complement receptors such as decay-activating factor, although this has not been definitely shown. These findings may be relevant for two reasons. First, they could be used in a very simplistic fashion to evaluate for endothelial activation. Secondly, they could explain non-specific signal enhancement when performing molecular imaging for specific cell endothelial cell surface receptors and lead to improvements in targeted probe design.

3. Ligand-targeted imaging of myocardial inflammation

The detection of mild or chronic inflammation with CEU has in general been performed by administration of microbubbles that bear antibodies or other ligands to endothelial cell adhesion molecules. Agents that have been used to image inflammation with CEU include those targeted to selectins, ICAM-1, VCAM-1, and MadCAM-1. The surface density of targeting ligands can affect attachment efficiency. Most agents have been designed with a ligand surface density of several 1000 per square micron of microbubble shell surface area which is generally well above that needed for maximum binding at microcirculatory shear stresses (<2 dyn/cm²). The binding properties of these agents under various shear stresses have been examined by flow chamber evaluation of microbubble attachment to activated endothelial cells grown in culture. According to these studies, microbubble attachment to endothelial cells decreases with increasing shear forces.

Figure 2

Figure 2 Targeted molecular imaging of leucocyte recruitment in canine myocardial ischaemia–reperfusion injury after transient occlusion of the left anterior descending (A–C) or left circumflex (D–F) coronary arteries, and spatial region of leucocyte-targeted myocardial contrast echocardiography in relation to the risk area and infarct area (G). Mid-ventricular short-axis images were obtained in vivo with leucocyte-targeted microbubbles 5 min after reflow (A and D), and ex vivo with radionuclide imaging of a Tc-labelled tracer (Tc-RP517) targeted to a leukotriene receptor (B and E). The location of inflammation by targeted ultrasound imaging was larger than with radionuclide imaging or the TTC-defined infarct region (C and F). Depicted by the graph, the area decreased over time indicating the ability of the ultrasound technique to detect low-grade transient inflammatory cell recruitment in the injured but non-infarcted risk area. The arrows denote a region of infarction in the papillary muscle with very early microvascular no-reflow where targeted ultrasound contrast agents do not enter. Colour scale at bottom from blue (lowest) to violet (highest). Reproduced with permission.
detecting the switch from fatty acid to glucose metabolism in ischaemic or recently ischaemic myocytes. Interest in ultrasound-based probes for ischaemic memory imaging stems from the need for a rapid technique that can be used at the bedside in most emergency departments. For this application, P-selectin has been proposed as a target since it is rapidly externalized on the endothelial surface from secretory granules in response to only mild ischaemia, and because upregulation can persist for hours after ischaemic injury. The ability of P-selectin-targeted microbubbles to adhere and produce robust CEU signal enhancement in post-ischaemic tissue was first demonstrated after renal ischaemia–reperfusion in mice. Subsequently, it has been shown that microbubbles targeted to P-selectin by surface conjugation of either selective antibodies or glycoprotein analogues to P-selectin glycoprotein ligand-1 produce robust myocardial signal enhancement in mice and rats after brief myocardial ischaemia (Figure 3). A clinically relevant feature of these studies was that the degree of ischaemia was not severe enough to produce infarction or prolonged stunning.

CEU with endothelial cell adhesion molecules (ECAM)-targeted microbubbles also has been tested as part of an effort to develop more sensitive non-invasive methods for early detection of heart transplant rejection. In early studies, sub-acute rejection of strain-mismatched of heterotopic cardiac allografts in rats could be detected non-invasively by ultrasound contrast enhancement following an intravenous injection of ICAM-1-targeted microbubbles (Figure 4). These data could support further investigation with any number of different ECAMs that are upregulated in transplant rejection and/or allograft vasculopathy which are both difficult to detect non-invasively until they are at an advanced stage.

It has been firmly established that the inflammatory response also plays a critical role in promoting ischaemia-related vascular remodelling (angiogenesis and arteriogenesis). As described in an accompanying review article in this series on angiogenesis imaging, targeted CEU in ischaemic limb models has recently been used to evaluate spatial-temporal patterns of endothelial cell activation (VCAM-1 expression) and monocyte recruitment in arteriogenesis.

4. Inflammation in atherosclerosis

The ability to image inflammatory changes in large vessels may provide unique information that can be used to assess risk in atherosclerosis. This capability could potentially be used to detect high-risk features in those with established disease, or possibly even to detect disease at a very early stage long before clinical manifestations arise. Unlike radionuclide or optical imaging molecular imaging probes that are usually diffusible, it is not possible to target microbubble agents to high-risk components of atherosclerosis that reside within the neointima such as oxidized lipids, cellular

![Figure 3 Ischaemic memory imaging with myocardial contrast echocardiography (MCE) and P-selectin targeted microbubbles. Intravital microscopy (A and B) demonstrates venular attachment of fluorescently labelled microbubbles to cremaster muscle exposed to brief ischaemic injury. Short-axis MCE images from a mouse illustrate the risk area (arrows) during brief occlusion of the LAD (C) which did not produce infarction, and the region of enhancement with P-selectin-targeted microbubbles 45 min after reflow (D). Colour scale at bottom. Reproduced with permission.](https://academic.oup.com/cardiovascres/article-abstract/84/2/182/325815)
apoptosis, or leukocyte-derived proteases and oxidative products. Instead, CEU evaluation of inflammatory phenotype in atherosclerosis has relied on targeting of ECAMs. Upregulation of ICAM-1, VCAM-1, and P-selectin participate in the recruitment of monocytes and lymphocytes in atherosclerosis and have been implicated in lesion initiation, progression, and susceptibility to acute atherothrombotic events.\textsuperscript{29–31} However, CEU imaging of ECAM expression in atherosclerosis is contingent on the ability of ultrasound contrast agents to adhere to the vascular endothelium in vessels with high-shear stresses. In this circumstance, adhesion occurs because of the pulsatile nature of flow in large vessels. Flow chamber studies have indicated that microbubbles targeted to the endothelium in large vessels adhere during low-shear diastole and can withstand very high shear during systole.\textsuperscript{21}

Targeted atherosclerosis imaging with ultrasound was first described with acoustically active sub-micron liposomes composed of multilamellar lipid vesicles that contain a small amount of entrapped air.\textsuperscript{32} In swine models of carotid atherosclerosis and injury, intra-arterial injection of ICAM-1 and VCAM-1-targeted immunoliposomes produced focal mural signal enhancement during high-frequency ultrasound examination.\textsuperscript{17,31} With microbubble targeting of VCAM-1, contrast agent attachment to atherosclerotic lesions and CEU signal enhancement of the aorta of Apo-E deficient mice has been possible with intravenous injection.\textsuperscript{21} In these studies, the intensity of VCAM-1-targeted signal enhancement was able to differentiate the degree of underlying plaque inflammatory status which was modulated by diet (\textit{Figure 5}). This wide variation in values in these experiments probably reflects issues with heterogeneous manifestation of disease in these models, and spatial resolution whereby the actual volume of tissue within the beam can influence intensity measurements. The latter issue will be resolved when large animal models of disease are studied.

Recent studies in mice deficient for the LDL receptor and the Apo-B mRNA editing protein have demonstrated that imaging endothelial expression of VCAM-1 or P-selectin with targeted microbubbles can detect the initial stages of intimal xanthoma formation.\textsuperscript{33} These results indicate that it may be feasible to use targeted ultrasound molecular imaging to identify aggressive disease at its earliest stage.

5. Limitations of the method

There are limitations of CEU methods for molecular imaging that will influence how the technology will be used in the clinical or research setting. The sensitivity of the technique for the detection of contrast agents is not a major obstacle. However, specificity of the technique remains a challenge because of the potential to generate signal from non-specific interactions between microbubbles and either leukocytes or activated endothelium. The recognition of the biological underpinnings for cell–shell interactions will probably provide solutions to this problem with further chemical modification of the shell. It is worth noting that for any application where the purpose is to simply detect an acute inflammatory process, any added non-specific retention to leukocytes could be enhance diagnostic potential.

With certain exceptions,\textsuperscript{26} in vivo targeting of microbubble agents to specific ECAMs has relied on using monoclonal Abs as a surface ligand. Many of the small molecule ligands for integrins and members of the immunoglobulin
superfamily of adhesion molecules that are widely available lack specificity. However, a mAb targeting strategy has disadvantages, such as the low on-rate for antibody–antigen reactions that can potentially limit microbubble attachment in high-velocity high-shear vessels. Refinement of microbubble targeting technology in the coming years will probably involve the use of high-specificity small molecule targeting ligands as they are discovered. Novel methods for molecular imaging with targeted agents will also be needed for clinical application. The ability to generate robust molecular imaging signal with low-mechanical index imaging that does not disrupt microbubbles is a subject that is currently under investigation.

Finally, there is the issue of whether the technique is sufficiently quantitative or provides more of a dichotomous answer (presence vs. absence of disease). CEU relies on the attachment of a multivalent particle. Similar to the binding of leucocytes in regions of inflammation, there is probably a threshold amount of target molecule expression that is needed for efficient binding. However, the quantitative capability of the technique has been corroborated by comparing intravital microscopy observations of the extent of leucocyte-targeted or P-selectin-targeted microbubble attachment with both leucocyte recruitment (rolling kinetics or adhesion density) and signal intensity under different pro-inflammatory conditions.

Figure 5 Targeted contrast ultrasound imaging of VCAM-1 expression in the aortic arch in a mouse model of atherosclerosis (Apo-E−/− mice). Contrast ultrasound images of the aortic arch were obtained after injection of VCAM-1-targeted microbubbles in a control wild-type mouse (A) and an Apo-E−/− fed a high-fat diet (HFD) (B) (colour scale at bottom). (C and D) Immunohistology of the aortic arch from an Apo-E−/− mouse on HFD illustrates the presence of a large lumen-encroaching plaque with positive VCAM-1 peroxidase staining within the plaque and along the vascular endothelium. (E) Quantitative video intensity data (box-whisker plots) demonstrating selective signal enhancement with VCAM-1-targeted microbubbles, the degree of which increased with severity of disease. Reproduced with permission.
6. Future directions and summary

Molecular imaging with contrast ultrasound is a rapidly developing field that has already been proven to be a useful research tool in animal models.25 The technique is well suited for evaluating inflammatory disease, since many of the key regulatory steps in the immune response take place within the vascular compartment. In this regard, ultrasound contrast agents have been developed that are able to assess immune cell recruitment and endothelial activation in acute and chronic inflammatory processes. Feasibility for CEU molecular imaging of atherosclerosis, ischaemia, and transplant rejection has been performed in relevant animal models of disease. Unfortunately, there has been essentially no experience using targeted ultrasound imaging probes in humans. There are several important challenges ahead that will determine whether the technique will make the transition to a clinical tool. Ultrasound contrast agents will need to be developed with ligands and surface conjugation strategies with proven safety and efficacy in humans. Equally important, there must be sufficient justification for the use of targeted contrast agent technology. Molecular evaluation must provide some useful incremental value to the established methods that are currently in use. This issue has already begun to be explored with radionuclide tracers for detecting recent myocardial ischaemia,22 and positron emission tomography probes for inflammatory cell activity in atherosclerosis.26 These data may forge a path for the potential application of an ultrasound-based approach which has many clinical advantages in terms of cost, speed, and availability.

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