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Molecular Imaging of Myocardial Injury: A Magnetofluorescent Approach

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Abstract

The role of molecular imaging in enhancing the understanding of myocardial injury and repair is rapidly expanding. Moreover, in recent years magnetic resonance and fluorescence-based approaches have been added to the molecular imaging armamentarium and have been used to image selected molecular and cellular targets in the myocardium. Apoptosis, necrosis, macrophage infiltration, myeloperoxidase activity, cathepsin activity, and type 1 collagen have all been imaged *in vivo* with a magnetofluorescent (MRI and/or fluorescence) approach. This review highlights the potential of these and other magnetofluorescent agents, with particular focus on their role in ischemic heart disease.

Introduction

Nuclear imaging techniques such as single photon emission CT (SPECT) and positron emission tomography (PET) have been used for many years to image molecular pathology in the myocardium [1•]. More recently, magnetic resonance and fluorescence-based techniques have been added to the molecular imaging armamentarium [2•,3], and a growing body of work with these modalities in the imaging of myocardial injury has emerged [4]. The use of magnetic, fluorescent, or combined magnetofluorescent platforms has added several new dimensions to molecular imaging of the myocardium: *in vivo* images can now be correlated with flow cytometry and fluorescence microscopy of the imaging agents, thus providing a detailed cellular understanding of probe uptake. In addition, activatable imaging agents can now be detected *in vivo*, providing a local assay of enzyme activity. Perhaps in no other area of the body, however, is the opportunity to integrate anatomic, physiologic, and molecular imaging into a single comprehensive imaging strategy as great as it is in the myocardium. The realization of this goal will undoubtedly be pursued via multiple strategies, each with its merits and disadvantages. This article highlights recent advances with magnetofluorescent imaging agents in the myocardium, focusing largely on ischemic heart disease and its sequelae.

Integrated Imaging Approach

The properties of molecular MRI and fluorescence imaging are highly complementary [4]. Physiologic parameters of interest in the myocardium include ventricular volumes, myocardial mass, ejection fraction, contractility, strain, perfusion, viability, and hemodynamics. Although at present fluorescence techniques are unable to image most of these parameters, MRI measures them with exceptional accuracy. Molecular MRI agents can also be detected *in vivo* with

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significantly better spatial resolution than that obtainable with noninvasive fluorescence techniques. However, fluorescence imaging agents provide excellent sensitivity (picomolar or better) and are ideally suited to activatable and multispectral (multi-target) approaches [3]. A compelling case can thus be made to, whenever possible, pursue a dual-modality magnetofluorescent approach for molecular imaging of the myocardium.

Magnetic nanoparticles such as gadolinium-loaded liposomes, gadolinium-containing micelles, and dextran-coated iron-oxide nanoparticles can be labeled with a fluorochrome without significantly changing the pharmacokinetics of the nanoparticle. Conventional gadolinium chelates, however, are frequently smaller than the fluorochrome and the pharmacokinetics of a joint construct can thus easily become dominated by the properties (size, charge, protein binding) of the fluorochrome. When fluorescent labeling is not feasible, molecular specificity in the myocardium can be confirmed using either scrambled versions of the probe or transgenic animal models (see collagen-binding and myeloperoxidase-activatable agents below).

Activatable Imaging Agents

One of the distinct advantages of both magnetic and fluorescent imaging agents is the ability to produce activatable imaging constructs with these platforms. While radioisotope-labeled probes emit radiation regardless of their state (they are always active), the fluorescence and magnetic properties of an imaging agent can be highly modulated. Activation of magnetofluorescent constructs can result from enzymatic cleavage of the agent into a more active form [5], or by assembly/disassembly of the agent [6]. To date, the latter strategy (assembly/disassembly) has been used successfully to image both activatable near-infrared (NIR) fluorochromes and novel gadolinium chelates in the myocardium [7,8•,9•].

Activatable NIR fluorochromes generally consist of two fluorescent moieties held in close proximity to each other by a peptide linker. In this configuration the fluorochromes quench each other, producing no fluorescence. Cleavage of the recognition sequence in the peptide linker by the specific enzyme of interest separates the two fluorescent moieties from each other, eliminating quenching and producing robust fluorescence [10]. Activatable magnetic resonance agents likewise rely on assembly/disassembly of the individual chelates or nanoparticles, which results in a detectable change in the magnetic relaxivity of the agent [6, 11•,12]. Importantly, both activatable magnetic and fluorescence imaging agents report the local activity of an enzyme of interest, and not merely its presence. This provides additional biological information to that obtained when labeled affinity ligands are used to bind to a particular site on an enzyme, which provides no information regarding the activity of the enzyme.

Pharmacokinetic and Technical Considerations

Molecular imaging of the myocardium requires the imaging agent to be small enough to penetrate the capillary membrane and enter the interstitial space. Magnetic nanoparticles, such as cross-linked iron oxide (CLIO), are 30 to 50 nm in size and able to penetrate both the normal and ischemic capillary membrane [13]. Smaller gadolinium-labeled liposomes and micelles are also able to do this, but larger liposomes remained confined to the intravascular space. Once in the interstitial space, the imaging agent then needs to remain inert and not bind nonspecifically to either cardiomyocytes or any of the surrounding connective tissue elements. CLIO and analogous iron-oxide nanoparticles are coated with dextran and thus remain inert and nonreactive in the interstitial space of the myocardium [14]. The potential for nonspecific binding of micelles and liposomes is of greater concern, and a potential disadvantage of the use of these agents in the myocardium.

The ideal blood half-life of an imaging agent designed for targeted myocardial imaging ranges from 1 to 4 hours. This provides adequate time for the agent to penetrate the capillary membrane and detect its molecular target, while minimizing background circulation of the probe. High concentrations of radiolabeled SPECT and PET imaging agents in the left ventricular blood pool or lungs can adversely affect the dynamic range of the imaging experiment [15], or require the imaging readout to be delayed. MRI, however, does not suffer from this limitation and the myocardial uptake of an agent can usually be accurately imaged independent of the blood pool signal. In addition, if required, the blood pool signal can be modified or nulled through the use of inversion prepulses, diffusion-encoded stimulated echo sequences, and other black blood techniques [14,16].

Fluorescence imaging agents that are not quenched in their baseline state can, like radioactive probes, produce a high signal in the blood pool. However, if background activation of a quenched fluorochrome in the blood pool is minimal, imaging of the signal generated by probe activation in the myocardium can be performed soon after the injection of the agent. The high hemoglobin concentration in the blood pool significantly attenuates light in the visible spectrum. Signal generated by a fluorochrome in the myocardium in the visible range of the spectrum may thus be significantly attenuated, precluding accurate detection [3]. The attenuation of light is lowest in the NIR portion of the spectrum, and in vivo fluorescent imaging of the myocardium is optimally performed in this range of the spectrum [3]. Background autofluorescence in the myocardium tends to be fairly high, particularly in recently infarcted myocardium. However, autofluorescence is also lowest in the NIR, providing additional impetus for the use of fluorochromes in this range of the spectrum [3]. The use of NIR fluorochromes, coupled with advances in detector technology and reconstruction algorithms, has allowed noninvasive three-dimensional fluorescence tomography of the myocardium to be performed in mice in vivo [8•,17•]. Localization of the fluorescent hot spot signal to the myocardium can be performed by simultaneously imaging a second blood pool agent, detecting the left ventricular blood pool through the creation of absorption maps, or by fusing the fluorescence image with an anatomical imaging modality such as MRI or x-ray CT [17•].

Magnetofluorescent Imaging in Ischemic Heart Disease

Ischemic heart disease remains a major public health problem despite advances in the primary and secondary prevention of atherosclerosis. Although major strides have been made in establishing and maintaining patency of the infarct-related artery, the prevention of reperfusion injury and heart failure remain problematic. Magnetofluorescent imaging of the molecular processes involved in myocardial ischemia, reperfusion, healing, and remodeling is being increasingly performed, and has the potential to greatly increase our understanding of these important processes.

Cardiomyocyte apoptosis and necrosis

Apoptosis is an energy-requiring process that ultimately leads to the activation of caspase-3 and programmed cell death [18]. During the first 4 to 6 hours of ischemia, apoptosis is the predominant form of cardiomyocyte loss [19]. In animal models of ischemia reperfusion, up to 25% of cardiomyocytes in the injured myocardium develop apoptosis, and inhibition of the process with pan-caspase inhibitors has been shown to improve cardiac function [20]. Early on in the apoptotic process, phosphatidylserine, a phospholipid normally present only on the inner surface of the membrane, becomes expressed on the outer surface of the cell membrane too. Most molecular imaging approaches to date have been directed at this process, namely the detection of phosphatidylserine on the outer cell membrane of apoptotic cells [18,21].

In a pioneering study, uptake of fluorescently labeled annexin in the myocardium was imaged with intravital microscopy in a mouse model of ischemia reperfusion [22]. Annexin is a small

protein that binds with high affinity and specificity to phosphatidylserine [21]. Maximal uptake of the agent was seen on the surface of the cardiomyocyte cell membrane within 20 to 25 minutes of reperfusion [22]. A technetium-labeled annexin construct was also developed and used to image cardiomyocyte apoptosis in patients with acute ischemia and transplant rejection [15,23]. The development of magnetic, magnetofluorescent, and second-generation fluorescent annexin constructs has been more recent and is still in the preclinical phase [24–26]. However, the advantages as well as challenges of these newer constructs are already apparent.

Annexin has been conjugated to long-circulating magnetic nanoparticles [24,26], gadolinium-loaded liposomes [27], and long circulating polymers decorated with NIR fluorochromes [25]. The use of NIR fluorochromes, rather than fluorochromes in the visible spectrum such as Oregon green or FIT-C, allows deep tissues such as the myocardium to be imaged noninvasively in vivo. Regardless of the imaging marker used, excessive modification of annexin during probe synthesis can completely inactivate it [24]. When appropriate conjugation chemistry is used, however, annexin constructs can be developed with a level of biological activity almost identical to that of unmodified annexin [24]. The largest experience with these constructs to date is with the magnetofluorescent agent AnxCLIO-Cy5.5 [24,26].

Cardiomyocyte apoptosis has been imaged in vivo with AnxCLIO-Cy5.5 in a mouse model of acute reperfusion injury (Fig. 1) [26]. No significant changes were seen in myocardial signal intensity when the mice were injected with an unlabeled control probe. However, as shown in Figure 1, injection of an identical dose (2 mg Fe/kg) of the annexin-labeled probe (AnxCLIO-Cy5.5) produced significant negative contrast enhancement in the injured myocardium [26]. T2* maps of the hypokinetic regions of the myocardium also showed significant differences in mice given the active versus unlabeled probe (Fig. 1). The in vivo MRI results were further confirmed by fluorescence reflectance imaging of the NIR fluorochrome (Cy5.5) on the probe ex vivo. The ability to integrate the molecular images of cardiomyocyte apoptosis with cine images of cardiac function allowed the extent and severity of cardiomyocyte apoptosis to be correlated directly with local myocardial contractility. In addition, the high spatial resolution of MRI allowed the transmural location and extent of cardiomyocyte apoptosis to be accurately assessed [26].

More recently, annexin-labeled liposomes have been used to detect apoptosis in an isolated rat heart after ischemia-reperfusion [27]. The small size of the liposome allowed it to penetrate into the interstitial space of the myocardium, but further experience will be needed with this construct to determine its efficacy in vivo. Ligands other than annexin have also been developed for the detection of surface components on apoptotic cells. The C2 domain of synaptotagmin and duramycin are examples of such ligands, but their use in the myocardium to date is limited or has involved nuclear imaging approaches [28]. The imaging of caspase-3 activity in the myocardium in vivo is an area of intense interest but will require significant additional research [18].

The cell membrane of apoptotic cardiomyocytes remains viable until late in to the apoptotic process. In contrast, cardiomyocytes undergoing necrosis are characterized by disintegration of the cell membrane, exposing intracellular proteins to imaging agents that are circulating in the extracellular space. This strategy was used to develop a radiolabeled anti-myosin antibody and to image cardiomyocyte necrosis in animal models and in patients [29,30]. Myosin is an intracellular protein that cannot be accessed by the anti-myosin antibody unless the cell membrane is compromised. The same anti-myosin antibody has also been conjugated to an iron-oxide nanoparticle and used to image cardiomyocyte necrosis in a rat model of acute ischemia [31]. Magnetofluorescent approaches to detect both cardiomyocyte apoptosis and necrosis have thus been developed and demonstrate excellent sensitivity in ischemic heart disease. In conditions such as heart failure, in which cardiomyocyte apoptosis occurs in a

significantly lower percentage of cardiomyocytes, longer echo times and higher doses of AnxCLIO-Cy5.5 need to be used. However, preliminary data suggest that a magnetofluorescent approach in this scenario is feasible as well.

Cellular infiltration of the myocardium

Several hours after ischemic injury, neutrophils begin to attach to adhesion molecules on injured endothelial cells and then to penetrate ischemic myocardium. This is followed by the development of a large macrophage infiltrate, beginning 24 to 48 hours after the insult. Magnetic and magnetofluorescent nanoparticles are taken up by macrophages and can be used to image the infiltration of these cells into the myocardium in vivo (Fig. 2). Several MRI-detectable nanoparticles have been used to do this, including iron-oxide nanoparticles [17•, 32], gadolinium-containing liposomes, and fluorine-containing liposomes [33•].

The accumulation of iron-oxide nanoparticles in macrophages infiltrating infarcted myocardium has been imaged with a conventional T2*-weighted approach as well as an off-resonance technique (Fig. 2) [17•, 34]. Off-resonance approaches generate positive contrast but have a lower sensitivity than T2*-weighted imaging and are more complex to perform at high field strengths [34]. Superparamagnetic iron-oxide nanoparticles become saturated above 0.5 Tesla and thus have equal sensitivity at clinical field strengths (1.5–3 T) and at the higher field strengths often used in preclinical studies (4.7–15 T). Gadolinium-based agents lose sensitivity at higher field strengths and are thus easier to image at clinical field strengths. Conversely, the sensitivity of fluorine-based agents is directly proportional to field strength, and detectability at high fields may thus not be reproducible at clinical field strengths.

Secretion of oxidative and degradative enzymes

Immune cells infiltrating the myocardium secrete degradative and oxidative enzymes such as myeloperoxidase (MPO), cathepsins, and matrix metalloproteases. MPO results in the generation of reactive oxygen species, which further exacerbate myocardial injury. Imaging of MPO activity in the myocardium in vivo has recently been performed using an activatable gadolinium-serotonin chelate (Fig. 3) [9•]. In the presence of MPO, the probe forms dimers and oligomers, which have a higher longitudinal relaxivity (R1) than the parent compound [11•]. Intravenous injection of the agent into wild-type mice, 48 hours after myocardial infarction, produced marked signal enhancement in the infarcted myocardium (Fig. 3). Homozygous MPO knockout mice showed no evidence of probe activation or signal enhancement in their infarcts, and heterozygous MPO knockout mice showed an intermediate level of signal enhancement in the infarct [9•]. Activation of the probe 24 hours after ischemia-reperfusion could also be detected but was reduced in mice treated with statins [9•]. The results of this study show that an activatable magnetic resonance contrast agent can be imaged in the myocardium in vivo, and that the activatable MPO agent studied possesses adequate sensitivity and dynamic range to detect a treatment effect.

In the first 2 weeks after myocardial infarction, macrophages play important roles in angiogenesis, wound healing, and the formation of an adequate scar. However, macrophages also have the potential during this healing phase to secrete degradative enzymes such as cathepsins and matrix metalloproteases, which break down the extracellular matrix and promote left ventricular remodeling. Activatable NIR fluorochromes sensitive to the detection of cathepsins and matrix metalloproteases have been developed and studied in the postinfarction setting (Fig. 4) [7, 8•]. Matrix metalloprotease activity in the myocardium was imaged serially ex vivo with fluorescence reflectance imaging after myocardial infarction. Fluorescence activity peaked 1 to 2 weeks postinfarction and persisted for 4 weeks [7]. Zymography showed that most of this signal was due to MMP2-mediated activation of the fluorochrome [7]. In a technical step forward, cathepsin activity postinfarction was imaged in

vivo with fluorescence tomography [8••]. Cathepsin activity was detected using the NIR fluorochrome ProSense680 (Fig. 4). ProSense (cathepsin) activity peaked on day 4 postinfarction, which was slightly earlier than the uptake of CLIO by macrophages, which peaked on day 6 postinfarction [8••]. Dual wavelength in vivo fluorescence tomography of two distinct fluorochromes (ProSense680 and CLIO-VT750) was performed in this study, demonstrating the multispectral capabilities of fluorescence imaging [8••].

Myocardial fibrosis and scar formation

The healed infarct is characterized by a fibrous scar, the transmural extent of which is inversely related to the potential for functional recovery in that segment of myocardium. Currently, the transmural extent of myocardial scar is determined with delayed-enhancement MRI, although this reflects the passive pharmacokinetics of gadolinium and not its molecular specificity for fibrosis. Recently, a small gadolinium chelate targeted to collagen type 1 has been developed and used to image myocardial fibrosis in the postinfarct setting (Fig. 5) [35]. The signal enhancement generated by the probe persisted for significantly longer than that generated by delayed enhancement imaging with a conventional unlabeled gadolinium chelate, and correlated well the histological distribution of fibrosis [36••]. Affinity for collagen type 1 was achieved through the use of a 16-amino acid peptide ligand [35]. Complete loss of uptake was seen when a scrambled version of the peptide was used (Fig. 5) [35]. Further study will be needed to clarify the potential role of this agent in the clinical setting. However, the experience with this probe demonstrates that highly expressed targets, such as collagen type 1, can be successfully imaged in vivo with conventional gadolinium chelates.

Conclusions

The role of magnetofluorescent molecular imaging is becoming rapidly established in preclinical investigation. The feasibility, safety, and value of molecular MRI in humans have also recently been demonstrated with a fibrin-detecting gadolinium chelate in 11 patients with suspected thrombosis [37••]. Ongoing clinical adoption of molecular MRI will be driven by several factors, including the more widespread use of cardiovascular MRI and the development of more complex and individualized therapies for cardiovascular disease, which will require careful monitoring and guidance. As new magnetofluorescent agents are developed, a careful balance will need to be achieved between approaches that provide adequate sensitivity while posing minimal potential for toxicity.

Clearly, translation of magnetofluorescent imaging agents into clinical practice will be more complex than that of radiolabeled nuclear imaging agents, which have higher sensitivity and can be administered in low amounts. The appeal of a magnetofluorescent approach, however, lies in the high spatial resolution of MRI as well as the ability to integrate molecular images with images of cardiac function, strain, perfusion, viability, and metabolism (spectroscopy). The myocardium thus provides an ideal setting for an integrated anatomic, physiologic, metabolic, and molecular imaging approach with MRI. It should be noted, moreover, that magnetic resonance–PET systems are currently being developed and will provide even more flexibility and functionality to that provided by MRI alone. Over the next decade it is thus likely that even further progress will be made in the field of magnetofluorescent molecular imaging, and in particular in the imaging of myocardial injury and repair in patients who have suffered a myocardial infarction.

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Figure 1.

Molecular MRI of cardiomyocyte apoptosis in vivo in a mouse model of ischemia reperfusion [26]. The magnetofluorescent nanoparticle AnxCLIO-Cy5.5 detects apoptotic cardiomyocytes in the injured hypokinetic anterior wall. **A**, T2*-weighted cine MRI showing negative contrast (signal hypointensity) in the anterior wall due to the accumulation of AnxCLIO-Cy5.5. **B**, In vivo T2* map in the hypokinetic myocardium of a mouse injected with AnxCLIO-Cy5.5. T2* values were significantly lower in the mice injected with AnxCLIO-Cy5.5 than in those injected with the unlabeled control probe [26]. (From Sosnovik et al. [26]; with permission.)

Figure 2.

Magnetofluorescent imaging of myocardial macrophage infiltration 96 hours after myocardial infarction. **A**, T2*-weighted MRI showing robust negative contrast in the infarcted myocardium due to the accumulation of CLIO-Cy5.5 in macrophages infiltrating the infarct [17••]. **B**, Imaging of iron-oxide-laden macrophages penetrating an infarct with a positive contrast off-resonance technique [34]. Only those protons in the vicinity of the magnetic nanoparticle are shifted off-resonance and hence detected. **C**, Fluorescence tomography of myocardial macrophage infiltration [17••]. The three-dimensional fluorescence dataset is acquired completely noninvasively in vivo. Two-dimensional slices at various depths through the animal are reconstructed and superimposed on a white light image of the mouse. A localized area of high fluorescence intensity is seen in a slice intersecting the infarcted myocardium. (From Sosnovik et al. [17••] and Farrar et al. [34]; with permission.)

Figure 3.

Molecular MRI of myeloperoxidase activity in infarcted myocardium [9••]. Activation of the gadolinium chelate by myeloperoxidase leads to polymerization of the agent and an increase in its detectability with a T1-weighted magnetic resonance sequence. The images shown are (A) an infarcted wild-type mouse, (B) infarcted heterozygous myeloperoxidase knockout mouse, and (C) infarcted homozygous myeloperoxidase knockout mouse. Strong activation of the agent is seen in the wild-type mouse, very little is seen in the homozygous knockout mouse, and an intermediate level of probe activation is seen in the heterozygous knockout. (*From Nahrendorf et al. [9••]; with permission.*)

Figure 4.

Fluorescence imaging of cathepsin activity in healing myocardial infarcts with the activatable near-infrared fluorochrome ProSense680 [8••]. Fluorescence tomography reveals the presence of elevated fluorescence intensity over the heart. The inset at the *bottom left* of the figure shows an ex vivo fluorescence reflectance image of the excised heart. Cathepsin-induced activation of the fluorochrome can be seen in the infarcted myocardium. (*From Nahrendorf et al. [8••]; with permission.*)

Figure 5. Molecular MRI of myocardial scar formation with a small gadolinium chelate targeted to collagen type 1 [35]. T1-weighted black blood images were acquired 40 minutes after the injection of the imaging agent. Hyperenhancement is seen in the infarct when the chelate labeled with the active peptide is injected, but not when a chelate labeled with a scrambled peptide is injected. (*From Caravan et al. [35]; with permission.*)