Published in final edited form as:

Pediatr Cardiol. 2011 March ; 32(3): 290–296. doi:10.1007/s00246-011-9894-y.

Cardiomyocyte Death: Insights from Molecular and Microstructural Magnetic Resonance Imaging

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Abstract

Cardiomyocytes can die via necrosis, apoptosis, and autophagy. Although the molecular signals and pathways underlying these processes have been well elucidated, the pathophysiology of cardiomyocyte death remains incompletely understood. This review describes the development and application of novel imaging techniques to detect and characterize cardiomyocyte death noninvasively in vivo. It focuses on molecular and microstructural magnetic resonance images (MRIs) and their respective abilities to image cellular events such as apoptosis, inflammation, and myofiber architecture. These in vivo imaging techniques have the potential to provide novel insights into the mechanisms of cardiomyocyte death and to help guide the development of novel cardioprotective therapies.

Keywords

Apoptosis; Diffusion; Molecular imaging; MRI; Myocardium; Necrosis

Introduction and Imaging Platforms

Over the past decade, noninvasive techniques have been developed to image processes in the cardiovascular system at the molecular and cellular levels [13,17]. These molecular imaging techniques have aimed to provide novel insights into the molecular pathophysiology of cardiovascular disease and to elucidate the impact of molecular events on whole-organ physiology. Among these techniques, molecular magnetic resonance imaging (MRI), discussed extensively in this review, has emerged as a powerful tool.

The advantages of MRI over nuclear-based molecular imaging techniques include its higher spatial resolution, its superior soft tissue contrast, and its ability to integrate molecular, anatomic, and physiologic imaging data [18]. In addition, MRI provides a unique platform

for imaging the microstructural architecture of the myocardium by imaging the diffusion of water [22]. Microstructural MRI provides insights into myocardial function at a scale between molecular and whole-organ imaging and has the potential to bridge further the divide between molecular biology and clinical translation. Molecular MRI has a sensitivity in the micromolar to nanomolar range depending on the imaging platform used. This is significantly lower than the picomolar sensitivity of nuclear-based and fluorescence imaging agents.

Another challenge with molecular MRI is that typical imaging agents, although well suited for imaging targets on the cell surface, cannot rapidly penetrate an intact cell membrane. In addition, once in the intracellular space, nanoparticulate MRI agents frequently are trafficked nonspecifically into endosomes and lysosomes. Although significant progress is being made in the development of intracellular MRI agents, nuclear imaging techniques remain superior in this regard.

Two broad imaging agent platforms are used for molecular MRI in the myocardium. Small gadolinium chelates, used as extracellular MR contrast agents for more than a decade, are well suited for detecting highly expressed targets such as fibrin and collagen [2,7]. These agents have detection thresholds in the micromolar range, which, although significantly superior to iodinated contrast agents, support only the detection of highly expressed targets. Novel gadolinium constructs have thus been developed to overcome this sensitivity limit including gadolinium-containing lipoproteins, liposomes, and micelles [1,25]. These large gadolinium constructs have nanomolar sensitivity but more complex pharmacokinetics and therefore are generally not well suited to imaging of the myocardium.

Magnetic iron oxide nanoparticles (MNPs) constitute the second large class of molecular MRI agents [19]. Their use as an MR platform is based on their ability to modulate the uniformity of a magnetic field. Because MNPs are superparamagnetic, they have high magnetic relaxivities, allowing for the detection of sparsely expressed targets in the nanomolar range. They also are small (<50 nm), can penetrate the capillary membrane, and remain inert in the interstitial space.

A variety of ligands can be conjugated to MNPs including fluorochromes, small molecules, peptides, and small proteins. Examples of such ligands include annexin for apoptosis imaging [14] and a vascular cell adhesion molecule-1 (VCAM-1)-targeted peptide for imaging adhesion molecule expression on the vascular endothelium [10].

Molecular MRI currently is playing a significant role in preclinical cardiovascular investigation. It has the potential for expansion to clinical investigation in the near future.

In the context of cell death, molecular MRI approaches have been developed to image cardiomyocyte apoptosis [15,20], cardiomyocyte necrosis [20], and the inflammatory response to cell death [12,16]. Techniques for imaging autophagy are being actively pursued. Microstructural MRI of ischemic injury in the myocardium has been performed ex vivo in a wide variety of models [22]. More recently, it has been performed in vivo in mice with ischemia-reperfusion injury. The application of these techniques in neonatal mice is more challenging but still feasible. Although significant technical challenges remain, as discussed later, molecular and microstructural MRI currently can provide valuable insights into the mechanism of cell death in the myocardium.

Molecular MRI of Cell Death

Cardiomyocytes can die via apoptosis, necrosis, or autophagy [8]. The complexity and cross talk between these processes are becoming increasingly well understood. Other manuscripts

in this volume deal with these extensively. For the purposes of imaging agent development, we exploit the fact that the cell membrane remains intact during apoptosis but becomes ruptured and permeable during necrosis. We further exploit the biologic (target) amplification inherent in the apoptotic cascade, whereby a small amount of activated intracellular caspase-3 leads to large amounts of phosphatidylserine expressed on the outer cell membrane [3]. It should be noted, however, that annexin binds to both phosphatidylserine on the outer membrane of apoptotic cells and phosphatidylserine on the inner membrane of necrotic cells. Two readouts are thus needed to resolve the mechanism of cell death. Fluorescent annexins frequently are used with vital dyes, such as propidium iodide, to distinguish apoptosis from necrosis.

Molecular MRI of cell death in the myocardium must thus address the following challenges: sensitivity and specificity to phosphatidylserine, ability to determine whether the binding of annexin is occurring on a cell with an intact or ruptured cell membrane, and ability to image these processes within the first 4 h of ischemia, during which apoptosis is most prevalent [3,9].

Molecular MRI of Apoptosis

Molecular MRI of apoptosis in the heart has been performed with the AnxCLIO-Cy5.5 nanoparticle. The synthesis of this agent has been described extensively [14]. It is smaller than 50 nm and has biologic activity similar to that of unmodified annexin. The superparamagnetic cross-linked iron oxide (CLIO) moiety on the probe provides an MRI signal, whereas the near infrared fluorochrome Cy5.5 permits fluorescence imaging and microscopy of the agent to be performed. Each AnxCLIO-Cy5.5 nanoparticle contains three to five annexin groups per CLIO, creating a multivalent platform with increased activity. Great care, however, must be taken during the synthesis of the nanoparticle to avoid inactivating the annexin moieties [14].

AnxCLIO-Cy5.5 has been used to image cardiomyocyte apoptosis in vivo in both acute ischemia [15,20], and chronic heart failure [21]. The latter scenario is significantly more challenging because cardiomyocyte apoptosis in heart failure is sparse at any given time point but persists over months and years. In addition, the capillary membrane in heart failure does not become leaky, making delivery of the agent to the interstitial space slower.

Strikingly different patterns of cardiomyocyte apoptosis have been seen in heart failure and ischemia-reperfusion. The ability to resolve these patterns in vivo and to correlate them with contractile function and molecular events at the local level underscores the value of molecular MRI.

An initial proof-of-principle study showed that AnxCLIO-Cy5.5 could successfully image cardiomyocyte apoptosis in vivo in a mouse model of ischemia-reperfusion [15]. In a follow-up study, imaging was performed during the first 4 h of reperfusion to document the evolution of cardiomyocyte apoptosis [20]. A version of AnxCLIO-Cy5.5 with an inactivated annexin moiety was used as the control nanoparticle. Both the active and control nanoparticles thus had identical sizes, charges, and blood half-lives (2.7–2.9 h).

Within 4 h of reperfusion, the control probe was washed out of the injured myocardium. In contrast, robust accumulation of the active probe was seen, producing signal hypointensity in the areas of myocardium where it had accumulated [20]. Cardiomyocyte apoptosis in ischemiareperfusion was most frequently seen in the midmyocardium (Fig. 1). In mice with mild to moderate wall motion abnormalities, cardiomyocyte apoptosis frequently was confined to the midmyocardium.

In contrast, in the mice with more severe injury, cardiomyocyte apoptosis began in the midmyocardium and thereafter spread transmurally [20]. The mechanism of this spatial distribution is not fully understood and requires further study. More work also is needed to determine whether similar patterns are seen in humans and large animals.

Molecular MRI of cardiomyocyte apoptosis in heart failure has been performed in postpartum Gaq-over-expressing mice [21]. A well-characterized cardiomyopathy developed in these mice, which by 2 weeks postpartum was characterized by apoptosis in 1–2% of cardiomyocytes [6]. The pattern of cardiomyocyte apoptosis seen with AnxCLIO-Cy5.5 in these mice was very different from that seen during acute ischemia. Cardiomyocyte apoptosis occurred in discrete isolated clusters in these mice [21], frequently in the subendocardium (Fig. 2). The uptake of AnxCLIO-Cy5.5 correlated strongly with caspase-3 activity in the myocardium. Molecular MRI of cardiomyocyte apoptosis in heart failure thus provides an excellent platform for further study investigating the pathophysiology of heart failure as well as the efficacy of novel therapies.

Simultaneous Imaging of Apoptosis and Necrosis

The importance of imaging cell membrane integrity in conjunction with the uptake of annexin has been alluded to earlier. A dual-contrast molecular MRI approach was thus developed to distinguish apoptosis from necrosis using AnxCLIO-Cy5.5 and a novel magnetofluorescent gadolinium chelate, Gd-DTPA-NBD [20]. Unlike many large and highly charged organic fluorochromes, NBD is small and without electric charge and did not significantly alter the pharmacokinetics of the gadolinium chelate, Gd-DTPA. Immunohistochemistry in a mouse model of myocardial infarction confirmed that Gd-DTPA-NBD did not cross intact cell membranes and that it accumulated only in areas of cell rupture and disintegration [20].

The developed dual-contrast MRI approach then was applied in a mouse model of ischemia-reperfusion (Fig. 3). The mice were injected with AnxCLIO-Cy5.5 at the onset of reperfusion, and T2*-weighted MR images were acquired within 4–6 h. The mice then were injected with Gd-DTPA-NBD, and delayed enhancement imaging was performed within 10–30 min.

The magnetic relaxivities of iron- and gadolinium-based contrast agents differ significantly at higher fields. This allowed the effects of AnxCLIO-Cy5.5 and Gd-DTPA-NBD on the proton pool to be separated and quantified. On the average, 21% of the myocardium with AnxCLIO-Cy5.5 accumulation showed delayed enhancement of Gd-DTPA-NBD [20]. More than 75% of the myocardium in which AnxCLIO-Cy5.5 accumulation was seen had membrane integrity, consistent with cardiomyocyte apoptosis (Fig. 3). The areas showing AnxCLIO-Cy5.5 accumulation and delayed gadolinium enhancement were indicative of cardiomyocyte necrosis.

The results of this study suggest that in a mouse model with 35 min of ischemia-reperfusion, the majority of apoptotic cardiomyocytes remain potentially salvageable within 4 h of injury. The fate of these myocytes, however, is not fully understood and requires further study.

Imaging Myocardial Inflammation

The role of inflammation in cardiomyocyte death after ischemia remains controversial. The generation of reactive oxygen species likely contributes to cardiomyocyte death, but trials of antiinflammatory therapies in ischemic heart disease have frequently yielded negative results. Molecular MRI thus has a significant role to play in efforts toward better

understanding the evolution and role of inflammation in myocardial injury. Enzymes involved in the generation of reactive oxygen species, such as myeloperoxidase, form an attractive target for molecular MRI. A myeloperoxidase (MPO)-activated MR imaging agent has been produced and used to image MPO activity in acute ischemia [12]. In the presence of MPO, the gadolinium-serotonin chelate forms dimers and oligomers with higher relaxivity and increased tissue retention. Statin therapy reduces MPO secretion and activation of the probe [12].

Macrophages do not play a role in acute cell death but do play an important role in the response to cell death, particularly necrosis. In the first few days after infarction, highly proteolytic lys6c-high macrophages infiltrate the infarct and remove the necrotic debris [11]. These macrophages can be imaged easily by MRI using macrophage-avid nanoparticles [16]. The proteolytic macrophage infiltrate transitions after a few days to a more proliferative lys6c-low macrophage population, which persists for approximately 2 weeks. Targeted imaging of cardiomyocyte death in inflamed and healing tissue frequently is complicated by nonspecific uptake of imaging agents. However, a vital DNA-binding gadolinium chelate (Gd-TO) developed recently is able to bind specifically to the DNA exposed by acutely necrotic cells [4], even in highly inflamed tissue. The use of Gd-TO in conjunction with AnxCLIO-Cy5.5 can thus be considered the *in vivo* molecular MRI analog of flow cytometry with fluorescent annexin and propidium iodide.

Microstructural Imaging

The myocardium has a highly intricate microstructure. Individual myocytes are arranged into fibers and groups of myofibers into sheets. It is the thickening and sliding of these myofiber sheets over one another that allows the myocardium to contract efficiently in systole. Landmark histologic studies by Streeter [24] showed that the myocardium comprises an array of crossing helical myofibers. Myofibers in the subendocardium form a positive or right-handed helix around the left ventricle, and subepicardial fibers form a negative or left-handed helix. Between the subendocardium and the subepicardium, fibers in the midmyocardium are circumferential. This architecture supports torsion and efficient contraction of the myocardium and frequently is perturbed in disease. Myocyte fibers and sheets can thus be regarded as the functional unit of the myocardium at a scale between that of the individual cardiomyocyte and the organ as a whole.

The use of MRI has the unique ability to resolve myocardial microstructure by imaging the diffusion of water [5]. Water diffuses most readily along the long axis of myofibers and hence can be used as an indirect measure of myofiber orientation. Numerous diffusion encoding and postprocessing schemes have been developed, and the interested reader is referred to several recent publications in the field [5,22]. In the context of cell death, measuring the mean diffusivity and fractional anisotropy of water diffusion is of significant value. However, it is the recently developed technique of diffusion tractography that is likely to provide the most compelling insights into cell death in the myocardium.

Diffusion tractography has traditionally been performed with diffusion tensor data sets. However, a more robust technique based on diffusion spectrum imaging (DSI) developed recently has provided novel insights into microstructural anatomy in ischemic heart disease [23].

A rat model of myocardial infarction was used. Permanent ligation of the left coronary artery was performed, and the infarcts were allowed to remodel for 3 weeks before imaging. The hearts then were perfused-fixed and excised for DSI-tractography, which was able to resolve both the normal myofiber anatomy and the complex microstructural patterns seen in infarcted myocardium (Fig. 4). The normal myocardium showed the characteristic crossing

helical structure described earlier. The myofibers in the midmyocardium were aligned circumferentially around the long axis of the ventricle (with a helix angle of zero), whereas the subendocardial fibers had a right-handed or positive helix angle, and the subepicardial fibers had a left-handed or negative helix angle. Imaging demonstrated that the transition of the helix angles from the endocardium to the epicardium occurred in a gradual and symmetric manner.

The myofiber anatomy in the infarct zones was markedly disrupted (Fig. 4). Numerous residual myofibers were present in the infarct and border zone [23]. Many of the residual myofibers were subendocardial despite the prevailing expectation that cardiomyocyte death would be most severe and complete in this zone. The infarct boundary did not resemble a smooth wave front, but rather, was highly irregular and characterized by numerous unevenly distributed residual myofibers. The residual subendocardial myofibers in the infarct frequently made contact, intersecting mid and subepicardial fibers remaining in the infarct and border zone. Numerous nodes of fiber contact were thus present in the infarct and border zone. The significance of these fiber patterns remains unclear. It is conceivable that this network of residual myofibers provides a mechanical benefit to the infarct. However, it is equally conceivable that these residual myofibers form a highly arrhythmogenic substrate and increase the potential for sudden cardiac death.

Conclusion

Molecular and microstructural imaging of cell death in the myocardium is in its nascency, but it already is providing valuable insights. The spatial patterns of cardiomyocyte apoptosis and necrosis identified with molecular and microstructural MRI suggest that the conventional wave front model of cell death frequently does not apply. Rather, it seems that the severity and mode of cell death are driven by a local set of factors that can differ markedly over short distances. Genomic and other traditional molecular biologic approaches will further elucidate these local factors over the coming years. Magnetic resonance imaging in all its forms (anatomic, functional, microstructural, and molecular) will play an important role in this endeavor and provide valuable complementary information.

Acknowledgments

This study was funded in part by R01 HL093038 (DES).

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Fig. 1. Molecular magnetic resonance imaging (MRI) of cardiomyocyte (CM) apoptosis in a mouse model of ischemia-reperfusion. **a** A mouse injected with the apoptosis-sensing agent AnxCLIO-Cy5.5. **b** A mouse injected with the inactivated control agent. The binding of AnxCLIO-Cy5.5 to apoptotic CMs produces signal hypointensity in the area of injury (yellow arrows). The pattern of AnxCLIO-Cy5.5 uptake shows that CM apoptosis (signal hypointensity) is occurring primarily in the midmyocardium. Reproduced with permission [20]

Fig. 2.

Molecular magnetic resonance imaging (MRI) of cardiomyocyte (CM) apoptosis in postpartum Gaq overexpressing mice. **a, b** T2*-weighted in vivo images. Numerous punctate foci of signal hypointensity are seen in the mouse injected with AnxCLIO-Cy5.5 (*white arrows* in **a**). **b** No such foci are seen in the mouse injected with the control probe. The in vivo findings are confirmed by ex vivo MRI (**d, e**) showing punctate foci of hypointensity in the mouse injected with the active probe (**d**) but not in the mouse injected with the control probe (**e**). Further confirmation of active probe uptake is provided by ex vivo fluorescence microscopy (**c**). The fluorescent signature of the probe can be seen on an apoptotic CM with characteristic blebs (*yellow arrows*). **f** Probe uptake *in vivo* can be quantified by constructing T2* maps. Reproduced with permission [21]

Fig. 3.

Dual-contrast molecular magnetic resonance imaging (MRI) of cardiomyocyte (CM) apoptosis and necrosis. Three slices from the mid left ventricle (LV) to the apex are shown. A T2*-weighted MRI of AnxCLIO-Cy5.5 is shown in the first column (**a**, **d**, **g**). Areas of myocardium with AnxCLIO-Cy5.5 uptake (*yellow arrows*) can be either apoptotic or necrotic. A second contrast agent is needed to resolve this. Delayed enhancement (DE) of Gd-DTPA-NBD (middle column, **b**, **e**, **h**) shows the areas of AnxCLIO-Cy5.5 uptake in which membrane rupture has occurred (*red arrows*). These areas with both AnxCLIO-Cy5.5 and Gd-DTPA-NBD uptake are necrotic. Histologic confirmation of AnxCLIO-Cy5.5 uptake can be confirmed with fluorescence microscopy (**c**) and Gd-DTPA-NBD uptake with immunohistochemistry (**f**). **I** At 4 h after 35 min of ischemia-reperfusion, more than 75% of apoptotic (AnxCLIO-Cy5.5-positive) CMs still are potentially viable (DE-negative). Reproduced with permission [20]

Fig. 4. Magnetic resonance imaging (MRI) tractography of rat hearts with the diffusion spectrum imaging (DSI) technique. The myofiber tracts are color coded by the helix or the spiral angle they make with the left ventricle. (**a–c**) The images in the upper row show a normal rat heart viewed in its short axis and from its lateral wall. In the normal myocardium, myofibers in the subendocardium and subepicardium have orthogonal helix angles and pass through separate transmural planes. Infarcted rat hearts are shown in the bottom row. The fiber architecture in the infarcts is severely perturbed, but numerous residual myofibers are present in the infarcts and their border zones. This can be seen with both MRI tractography (**d, e**) and histology (**f**). Magnetic resonance tractography further shows that the residual subendocardial and subepicardial fibers frequently make direct contact with each other. Reproduced with permission [23]