Molecular MRI Detects Low Levels of Cardiomyocyte Apoptosis in a Transgenic Model of Chronic Heart Failure

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Background—The ability to image cardiomyocyte (CM) apoptosis in heart failure could facilitate more accurate diagnostics and optimize targeted therapeutics. We thus aimed to develop a platform to image CM apoptosis quantitatively and specifically in heart failure in vivo. The myocardium in heart failure, however, is characterized by very low levels of CM apoptosis and normal vascular permeability, factors thought to preclude the use of molecular MRI.

Methods and Results—Female mice with overexpression of Gaq were studied. Two weeks postpartum, these mice develop a cardiomyopathy characterized by low levels of CM apoptosis and minimal myocardial necrosis or inflammation. The mice were injected with the annexin-labeled nanoparticle (AnxCLIO-Cy5.5) or a control probe (CLIO-Cy5.5) and imaged in vivo at 9.4 T. Uptake of AnxCLIO-Cy5.5 occurred in isolated clusters, frequently in the subendocardium. Myocardial T2* was significantly lower (7.6 ± 1.5 ms versus 16.8 ± 2.7 ms, P < 0.05) in the mice injected with AnxCLIO-Cy5.5 versus CLIO-Cy5.5, consistent with the uptake of AnxCLIO-Cy5.5 by apoptotic CMs. A strong correlation (r² = 0.86, P < 0.05) was seen between in vivo T2* (AnxCLIO-Cy5.5 uptake) and myocardial caspase-3 activity.

Conclusions—The ability of molecular MRI to image sparsely expressed targets in the myocardium is demonstrated in this study. Moreover, a novel platform for high-resolution and specific imaging of CM apoptosis in heart failure is established. In addition to providing novel insights into the pathogenesis of CM apoptosis, the developed platform could facilitate the development of novel antiapoptotic therapies in heart failure. (Circ Cardiovasc Imaging. 2009;2:468-475.)

Key Words: apoptosis ■ heart failure ■ MRI ■ molecular imaging ■ cardiomyocyte

Cardiomyocyte (CM) apoptosis plays an important role in the development and progression of heart failure,1,2 and molecular imaging of this process could thus facilitate the development of novel cardioprotective therapies. Molecular imaging of apoptosis is most frequently performed with annexin-labeled imaging agents, which detect phosphatidylserine on the apoptotic cell membrane.3,4 In a series of breakthrough cardiovascular studies, technetium-labeled annexin was used to image cell death in vivo in acute ischemia and transplant rejection.5,6 More recently, a magnetofluorescent annexin construct, AnxCLIO-Cy5.5, has been developed and used to image CM apoptosis in vivo in a mouse model of ischemia reperfusion.7

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The level of CM apoptosis in chronic heart failure, however, is substantially lower than that seen in acute conditions such as ischemia and transplant rejection.1,2,8,9 In addition, unlike acutely injured or inflamed tissues, the capillary membrane in chronic heart failure does not become hyperpermeable, potentially reducing the amount of the imaging agent that can be delivered to the interstitial space and the apoptotic CMs. These challenges are particularly relevant to molecular MRI, which involves the use of larger agents than nuclear imaging10 and has a significantly lower sensitivity. The use of molecular MRI to image CM apoptosis, however, is particularly compelling given the unparalleled ability of MRI to image myocardial structure, function, and viability.10

The primary aim of this study was to determine whether molecular MRI could be used to image low levels of CM apoptosis in a mouse model of chronic heart failure. Postpartum mice with 5-fold overexpression of the Gaq transgene were imaged with the apoptosis-sensing nanoparticle AnxCLIO-Cy5.5. These Gaq-overexpressing mice develop a well-described postpartum cardiomyopathy characterized by low levels of CM apoptosis (1% to 2%) in its chronic phase,
minimal myocardial inflammation and necrosis, and normal capillary permeability. We demonstrate in the study that in vivo uptake of AnxCLIO-Cy5.5 correlates strongly with myocardial caspase-3 activity, demonstrating the sensitivity and specificity of AnxCLIO-Cy5.5 for a sparse population of purely apoptotic CMs. A new platform and readout for basic and translational research of CM apoptosis in heart failure is thus established.

Methods

Generation of the Model

Heterozygous FVB/N mice with 5-fold overexpression of the Gaq transgene were kindly provided by Dr Gerald Dorn. Genotypic characterization of the female pups was performed with a real-time quantitative polymerase chain reaction system after purifying genomic DNA from the tail. Male mice not needed to maintain the line were euthanized at birth. Heterozygous female pups were housed until 3 months of age, at which time they were mated with wild-type males. Postpartum females were identified on the day of delivery and imaged 10 to 14 days after delivery. Whereas higher levels of CM apoptosis have been documented in the early postpartum period (days 1 to 4), by 10 to 14 days postpartum apoptosis is seen in only 1% to 2% of the CMs in this model. Twelve postpartum mice were imaged in 2 phases: In the initial phase, ex vivo fluorescence reflectance imaging (FRI) was performed in 6 postpartum Gaq mice to demonstrate feasibility and proof of principle. In the second phase, in vivo molecular MRI and ex vivo MRI and FRI were performed in 10 postpartum Gaq mice, and the imaging data were correlated with myocardial caspase-3 activity and levels of cleaved PARP-1.

Phase 1: Ex Vivo FRI

Postpartum Gaq mice were injected (tail vein) with 3 mg Fe/kg of AnxCLIO-Cy5.5 (n=3) or the unlabeled control probe CLIO-Cy5.5 (n=3). The properties of AnxCLIO-Cy5 have been previously described, although it should be noted that the transrelaxivity of the current agent is >80 mmol/L s⁻¹. AnxCLIO-Cy5.5 is <50 nm in size and has a biological activity similar to that of unmodified annexin. The superparamagnetic cross-linked iron oxide (CLIO) moiety on the probe provides an MRI readout, whereas the near infrared fluorochrome Cy5.5 allows fluorescence imaging and microscopy of the agent to be performed. FRI was performed 48 hours after injection of the imaging agent, providing a low-resolution but inexpensive and rapid (5 minutes per heart) screen of the pharmacokinetics and target organ uptake of AnxCLIO-Cy5.5. The blood half-life of AnxCLIO-Cy5.5 in mice is ~3 hours, whereas that of CLIO-Cy5.5 is ~10 hours. An imaging time point 48 hours after injection was chosen to ensure that both agents, particularly CLIO-Cy5.5, had been completely cleared from the blood pool. Selection of this time point ensured that any differences in myocardial signal between the 2 probes would reflect the ability of AnxCLIO-Cy5.5 to cross the capillary membrane, enter the interstitial space, and bind to apoptotic CMs.

Before FRI, the excised hearts were bisected in the short axis at the midventricular level. FRI was performed using a high-performance digital CCD camera (Sensicam, Cooke Corporation, Auburn Hills, Mich) in reflection mode. The fluorescence images were obtained using a 672-nm diode laser, an excitation strength of 550 mA, a 60-second exposure time, and a Cy5.5 filter. The fluorescence signal in both portions of each heart was averaged and mapped linearly. FRI data from phase 1 of the study were pooled for the FRI data from phase 2 (see below). The fluorescence intensity in the mice injected with AnxCLIO-Cy5.5 and CLIO-Cy5.5 was then compared using a Mann–Whitney test (Prism, Graphpad, La Jolla, Calif).

Phase 2: In Vivo MRI

The 10 mice in the second phase of the study were injected with 10 mg Fe/kg of AnxCLIO-Cy5.5 (n=5) or CLIO-Cy5.5 (n=5). In vivo MRI was performed 48 hours after probe injection on a horizontal bore, 9.4-T small-animal MRI scanner (Biospec, Bruker, Billerica, Mass) with cardiorespiratory gating (SA instruments, Stonybrook, NY) and a cardiac-tailored surface coil. Gradient echo images were acquired from the base to the apex of the heart to quantify left ventricular volumes and ejection fraction (EF). The following imaging parameters were used: field of view, 30×30 mm; slice, 1 mm; matrix, 200×200 (150 μm resolution); flip angle, 30°; echo time (TE), 2 ms; 16 frames/R-R interval, Nex 4.

End-diastolic and end-systolic volumes were measured offline in each slice using a freeware Dicom reader (Osirix, University of Geneva, Geneva, Switzerland) and summed to calculate left ventricular end-diastolic volume, end-systolic volume, and EF. Midsystolic T2* maps were created in the short axis of the left ventricle, at the midventricular level, from gradient echo images acquired with the parameters above and at TE of 3.5, 5.0, 6.5, and 8 ms. T2* values were calculated using a monoexponential decay model, as previously described. Susceptibility artifacts from the lungs precluded accurate interpretation of myocardial T2* values over the inferior portion of the left ventricle, and these areas were thus excluded from the analysis. In vivo T2* values in the mice injected with AnxCLIO-Cy5.5 and CLIO-Cy5.5 were compared using a Mann–Whitney test (Prism, Graphpad, La Jolla, Calif).

Measurement of Myocardial Caspase-3 Activity and Cleaved PARP-1

After MRI, the mice were euthanized and the excised hearts were bisected at the midventricular level for further analysis. The basal half of the left ventricle was flash-frozen and used to assess myocardial caspase-3 activity and levels of cleaved PARP-1, whereas the apical half was embedded for microscopy. Caspase-3 activity was measured with an assay based on the cleavage of a fluorogenic DEVD-AMC substrate. A Western blot assay was performed to measure levels of cleaved PARP-1, which reflects the downstream activity of caspase-3. Recent reports, however, suggest that PARP-1 can be cleaved during autophagy as well. Left ventricular EF and the in vivo uptake of AnxCLIO-Cy5.5 (T2* values) were correlated with caspase-3 activity and PARP-1 using both Pearson and Spearman correlations (Prism).

The Western blot assay for cleaved PARP-1 was performed with a commercially available antibody for cleaved PARP-1 (Santa Cruz Biotech, Santa Cruz, Calif). The gels were then stripped with Restore buffer (Pierce, Rockford, Ill) and, if possible, stained again with an anti-GAPDH (glyceraldehyde-3-phosphate) antibody (Rockland Immuno-chemicals, Gilbertsville, Pa). If not possible, then a separate gel was run for the loading controls. Blots were developed with Western Lightning chemiluminescence reagent (PerkinElmer, Waltham, Mass) and molecular weights were compared with bands for Precision Plus Protein WesternC standards (BioRad, Hercules, Calif). Densitometry was performed with a custom macro executed in ImageJ software to treat all samples identically. The fluorogenic readout of caspase-3 activity involved diluting homogenized myocardial samples in a pH 7.4 buffer containing 100 mol/L 7-amino-4-methylcoumarin–derived caspase-3 substrate (DEVD-AMC). An AMC standard curve was used to convert arbitrary fluorescent units into moles of product AMC generated. Fluorescence measurements (excitation, 342 nm; emission, 441 nm) were made with a fluorescence plate reader both before and after a 6-hour incubation with substrate. Bicinchoninic acid protein assays (Pierce, Rockford, Ill) were performed to determine the total protein concentration in each
heart sample and used to normalize the fluorescence signal per mg of tissue.

Ex Vivo MRI and FRI and Histology

Ten histological sections, each 5 μm thick with 10-μm gaps, were obtained from the midventricular portion of each embedded heart. The remaining portion of the myocardium was allowed to thaw, rinsed in PBS, and then placed in batches of 2 in a fluorocarbon matching medium for ex vivo MR microscopy. MR microscopy was performed at 9.4 T using a tailored solenoid radiofrequency coil and a 3D gradient echo sequence with an isotropic spatial resolution of 65 μm and a flip angle of 30°. Two data sets, each taking 55 minutes, were obtained with TEs of 2.9 and 8 ms, respectively. FRI of the myocardium was then performed to corroborate the MRI and prior (phase 1) FRI findings.

CM apoptosis was identified on the histological sections with a terminal uridine nick-end labeling (TUNEL) assay (Integreen, New York, NY). The sections were counterstained with blue hematoxylin, and commercially available human lymph node sections provided positive controls. Fluorescence microscopy of AnxCLIO-Cy5.5 uptake was performed using an upright epifluorescence microscope (Eclipse 80i, Nikon Instruments, Melville, NY) with the following filters: excitation, 650±22.5 nm; emission, 680 nm long pass and 710±25 nm bandpass.

Averaged results throughout this report are reported as mean±SEM. A probability value of <0.05 was regarded as significant in the Mann–Whitney and correlation tests. All experiments were performed in accordance with the guidelines for the humane care of research animals at our institution.

Results

Pooled analysis of the FRI data from both phases of the study (Figure 1) showed a significantly greater fluorescence signal (7.6±0.3 versus 6.1±0.3, P<0.05) in the mice injected with AnxCLIO-Cy5.5 (n=8) than in those injected with CLIO-Cy5.5 (n=8). Representative in vivo MRIs from phase 2 of the study are shown in Figure 2. Myocardial T2* values were significantly lower in the mice injected with AnxCLIO-Cy5.5 than in those injected with CLIO-Cy5.5 (7.6±1.5 ms versus 16.8±2.7 ms, P<0.05). T2*-weighted images at a TE of 8 ms are also shown in Figure 2. Multiple discrete foci of signal hypointensity, consistent with probe uptake, could be seen in the mice injected with AnxCLIO-Cy5.5 but not in those injected with CLIO-Cy5.5. Uptake of the probe was irregular,
patchy, and frequently most prominent in the subendocardium (Figure 2).

Only those mice with an EF >60% were included in the comparison of AnxCLIO-Cy5.5 and CLIO-Cy5.5 accumulation. This ensured that the analysis of probe uptake was performed between 2 groups of mice with similar phenotypes. Nine of 10 of the Gaq mice had an EF >60% (mean, 49 ± 2%) and were thus included in the analysis. The EF of 1 of the mice injected with AnxCLIO-Cy5.5, however, was fully preserved at 68%, and this mouse was thus excluded from the uptake comparison of AnxCLIO-Cy5.5 and CLIO-Cy5.5 (Figure 2).

Ex vivo MRI of the excised hearts confirmed the in vivo findings. Multiple discrete areas of signal hypointensity, consistent with probe uptake, were seen in the mice injected with AnxCLIO-Cy5.5 but not in those injected with CLIO-Cy5.5 (Figure 3). Fluorescence microscopy of the mice injected with AnxCLIO-Cy5.5 revealed that the probe was bound to the cell membrane of apoptotic CMs, many showing gross morphological features of apoptosis such as blebbing (Figure 3). Uptake of AnxCLIO-Cy5.5 occurred in discrete isolated foci, each consisting of 1 to 5 apoptotic CMs. No evidence of CLIO-Cy5.5 uptake was seen by fluorescence microscopy.

Levels of GAPDH were similar in all mice (Figure 4). Likewise, no significant difference in myocardial caspase-3 activity was seen between the 2 groups of mice. However, presumably because of random variation in the model, levels of cleaved PARP-1 were significantly lower (0.49±0.08 versus 0.84±0.04, P<0.005) in the mice injected with AnxCLIO-Cy5.5 and spanned a large range (Figure 4). In the mouse with the fully preserved EF (68%), the level of cleaved PARP-1 was barely higher than that seen in healthy wild-type mice. Caspase-3 activity in this mouse was also significantly lower than any of the other 9 mice in phase 2 of the study. TUNEL staining in all mice revealed only occasional apoptotic CMs (Figure 4), even in those mice with the highest levels of myocardial caspase-3 activity. Although higher
levels of apoptosis are seen in this model in the first 4 postpartum days, similarly low levels (1% to 2%) of apoptosis have been previously reported in this model 2 weeks postpartum.

A strong correlation (Spearman \( r = 0.8 \), Pearson \( r = 0.93 \), and \( r^2 = 0.86 \), \( P < 0.05 \)) was seen in the mice injected with AnxCLIO-Cy5.5 between the in vivo T2* values, reflecting the degree of probe uptake and myocardial caspase-3 activity (Figure 5). Likewise, a strong correlation (Spearman \( r = 0.87 \), Pearson \( r = 0.92 \), and \( r^2 = 0.85 \), \( P < 0.0005 \)) was seen between EF and levels of cleaved PARP-1 (Figure 5). The strength of this correlation (EF and PARP-1) is also demonstrated in Figure 6, showing 2 mice injected with AnxCLIO-Cy5.5 with significantly different EFs and levels of cleaved PARP-1.

**Discussion**

Transgenic mouse models have shown that low but persistent levels of CM apoptosis can overwhelm the limited regenerative capacity of the myocardium and result in heart failure. The ability to serially image CM apoptosis in heart failure could thus facilitate more accurate diagnostics and the development of targeted therapeutics. We demonstrate in the current study that molecular MRI with the apoptosis-sensing nanoparticle AnxCLIO-Cy5.5 can noninvasively image low levels of CM apoptosis in a transgenic model of heart failure. Moreover, we show that in vivo quantification of AnxCLIO-Cy5.5 uptake correlates strongly with myocardial caspase-3 activity. A novel platform allowing integrated anatomic, physiological, and molecular imaging of CM apoptosis in heart failure is thus established.

Molecular imaging of apoptosis has most frequently exploited the ability of annexin V or the C2 domain of synaptotagmin to bind to phosphatidylserine on the apoptotic cell membrane. Pioneering work with a technetium-labeled annexin construct showed that cell death could be imaged in patients with acute ischemic syndromes and transplant rejection. More recently, the same construct has been used to image cell death in 8 patients with dilated cardiomyopathy. The etiology of the cardiomyopathy in these patients, however, was not defined, and biopsies to exclude myocarditis could not be performed. Nevertheless, the 4 patients who showed evidence of probe...
uptake had a more rapid decline in their clinical status, whereas the patients with no probe uptake remained stable.\(^1^9\)

Despite the success of these highly pioneering clinical studies, concerns have been raised that the use of annexin-based probes in the complex and multifaceted milieu of cardiovascular injury could reflect binding to both apoptotic and necrotic CMs,\(^2^0\) apoptotic macrophages,\(^2^1,2^2\) and nonapoptotic lymphocytes.\(^2^3,2^4\) The sensitivity and specificity of technetium-labeled annexin for a population of purely apoptotic CMs has thus been difficult to determine. In contrast, the transgenic mouse model used in this study is a controlled model of pure CM apoptosis with minimal CM necrosis or inflammation.\(^1^1,1^2\) The results of this study thus show that the binding of annexin to a population of purely apoptotic cells can generate adequate signal to be imaged noninvasively in vivo. This finding has important implications both for the utility of annexin as an imaging marker of CM apoptosis and more generally for the sensitivity of molecular MRI.

The correlation between AnxCLIO-Cy5.5 uptake and myocardial caspase-3 activity was extremely strong (Figure 5). The correlation between EF, however, was stronger with cleaved PARP-1 than with caspase-3 (Figure 5). This probably reflects a well-described phenomenon in CMs in which caspase-3 activation results in the rapid expression of phosphatidylserine on the cell membrane and the cleavage of some cytosolic and myofibrillar proteins,\(^2^8\) but the cell remains viable.\(^2^9,3^0\) It has been hypothesized that these myofibrillar CMs may represent an interrupted or forme fruste of CM apoptosis.\(^2^9,3^0\)

Complete execution of the apoptotic cascade with cleavage of PARP-1 and nuclear fragmentation may represent the response of the CM to a more severe insult, in which its upregulated prosurvival signals are overwhelmed.\(^3^0\) This would account for the strength of the correlation seen between EF and cleaved PARP-1. It should also be noted that whereas levels of cleaved PARP-1 largely represent its cleavage by caspase-3, recent reports suggest that autophagy can also result in the cleavage of PARP-1.\(^1^7\) Cleaved PARP-1 may thus be a composite marker of both caspase-3 activity (apoptosis) and autophagy, also explaining the strength of its correlation with EF.

The small size (<50 nm) and long circulation half-life of AnxCLIO-Cy5.5 allow it to move into the interstitial space via slow transport processes such as diffusion in a manner analogous to which large immunoglobulins and lymphotrophic nanoparticles reach the interstitial space.\(^3^1\) The ability of AnxCLIO-Cy5.5 to penetrate the interstitial space of the myocardium has been previously demonstrated\(^7\) but only in the setting of acute injury and increased vascular permeability.\(^7\) We now show that AnxCLIO-Cy5.5 is able to cross a normal capillary membrane, access the interstitial space of the myocardium, and bind to a target expressed on only 1% to 2% of CMs.\(^1^5\) The high affinity of AnxCLIO-Cy5.5 for apoptotic cells,\(^1^5\) its high magnetic relaxivity, and the stability of its signal over time allowed a detectable MR signal to be generated even though only a small fraction of the injected dose probably reached the interstitial space of the myocardium. The excellent sensitivity of molecular MRI shown in this study suggests that it could support the imaging of a variety of sparsely expressed targets in the myocardium, even in the presence of normal vascular permeability.

The uptake of AnxCLIO-Cy5.5 in the ex vivo images (and in the portions of the myocardium visualized in vivo) was seen in all regions of the left ventricle but was frequently most predominant in the subendocardium and in the anterior and lateral walls (Figures 2 and 3). In addition, uptake of the probe was patchy, consistent with the presence of scattered clusters of apoptotic CMs in the myocardium (Figures 2 and 3). Similar patterns of CM apoptosis have been documented in humans and in animal models of heart failure: Focal uptake of technetium-labeled annexin was seen in the anterolateral wall of the left ventricle in a patient with dilated cardiomyopathy and heart failure.\(^1^9\) Likewise, clusters and scattered groups of apoptotic CMs have been documented histologically in patients with dilated cardiomyopathy and heart failure,\(^5,9\) with the subendocardium most frequently involved.\(^8,3^2\) A strong correlation has been reported between wall stress, levels of the proapoptotic protein Bax, and CM apoptosis, all of which were highest in the subendocardium.\(^3^2\) Further study will be needed to elucidate the mechanisms underlying this spatial pattern of CM apoptosis and underscores the value of molecular MRI, which allows CM apoptosis and left ventricular mechanics to be imaged with high spatial resolution in a single integrated data set.\(^1^0\)

The dose of iron oxide used in this study was substantially lower than the doses used in many previous studies in mice.\(^3^3,3^4\) Moreover, the use of magnetic nanoparticles with similar sizes and properties to CLIO but significantly higher relaxivities should allow even lower doses of iron oxide to be used.\(^3^5,3^6\) The design of this study did not allow the tissue elimination of bound AnxCLIO-Cy5.5 to be determined, which, based on prior experience with analogous magnetic nanoparticles, could take up to 1 to 2 weeks.\(^3^7\) Nevertheless, elimination over 1 to 2 weeks would still allow serial imaging in a chronic condition such as heart failure to be performed at several physiologically meaningful time points.

In conclusion, a novel platform for basic and translational research of antiapoptotic therapies in heart failure is established in this study. The postpartum Gaq-overexpressing mouse recapitulates a variant of heart failure seen in humans and provides a highly pure and specific model of CM apoptosis. In addition to its implications for future research in heart failure, the current study also demonstrates the ability of molecular MRI to image sparsely expressed molecular targets in the myocardium, thus expanding the scope and potential applications of molecular MRI.

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Disclosures
Dr Weissleder is a consultant and shareholder in Visen Medical (Woburn, Mass).

References


**CLINICAL PERSPECTIVE**

In animal models and humans with heart failure, it has been shown that low but persistent levels of apoptosis result in the loss of large numbers of cardiomyocytes and hence the progression of heart failure. Molecular imaging of cardiomyocyte apoptosis in heart failure could help facilitate the development of novel therapies but has been difficult to perform in vivo without sacrificing either spatial resolution or sensitivity. In the current report, we report the use of a magnetofluorescent nanoparticle, AnxCLIO-Cy5.5, to image cardiomyocyte apoptosis in heart failure. We show in the study that the agent is able to cross the capillary membrane and successfully image apoptosis, involving only 1% to 2% of cardiomyocytes, in vivo. Moreover, the high resolution of MRI revealed that the apoptotic myocytes were present in scattered clusters, particularly in the subendocardium. A high correlation was seen between the uptake of AnxCLIO-Cy5.5 and levels of myocardial caspase-3. A new tool and platform has thus been developed for translational research in heart failure. Molecular imaging of apoptosis with this agent should allow the evaluation of apoptosis in heart failure and better the understanding of its relation to ventricular function. The use of AnxCLIO-Cy5.5 also provides a powerful tool to monitor the efficacy of novel antiapoptotic therapies. Although the immediate impact of AnxCLIO-Cy5.5 probably will be preclinical, translation of the agent into the clinical arena is highly feasible and is being actively pursued.
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