Molecular MRI of Acute Necrosis With a Novel DNA-Binding Gadolinium Chelate
Kinetics of Cell Death and Clearance in Infarcted Myocardium

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Background—Current techniques to image cell death in the myocardium are largely nonspecific. We report the use of a novel DNA-binding gadolinium chelate (Gd-TO) to specifically detect the exposed DNA in acutely necrotic (ruptured) cells in vivo.

Methods and Results—In vivo MRI was performed in 20 mice with myocardial infarction (MI). The mice were injected with Gd-TO or Gd-DTPA at varying time points after MI. MRI was performed 2 hours after probe injection, to avoid nonspecific signal from the late gadolinium enhancement effect. Cell rupture (Gd-TO uptake) was present within 2 hours of infarction but peaked 9 to 18 hours after the onset of injury. A significant increase in the longitudinal relaxation rate ($R_1$) in the infarct was seen in mice injected with Gd-TO within 48 hours of MI, but not in those injected more than 72 hours after MI ($R_1 = 1.24 \pm 0.08$ and $0.92 \pm 0.03$ s$^{-1}$, respectively, $P < 0.001$). Gd-DTPA, unlike Gd-TO, washed completely out of acute infarcts within 2 hours of injection ($P < 0.001$). The binding of Gd-TO to exposed DNA in acute infarcts was confirmed with fluorescence microscopy.

Conclusions—Gd-TO specifically binds to acutely necrotic cells and can be used to image the mechanism and chronicity of cell death in injured myocardium. Cell rupture in acute MI begins early but peaks many hours after the onset of injury. The ruptured cells are efficiently cleared by the immune system and are no longer present in the myocardium 72 hours after injury. (Circ Cardiovasc Imaging. 2011;4:729-737.)

Key Words: molecular imaging ■ MRI ■ necrosis ■ myocardium ■ infarction

The transition of cells from a healthy (vital) to an apoptotic or necrotic state is a feature of many cardiovascular diseases including ischemic injury, heart failure, and transplant rejection. Imaging cell death in vivo thus has the potential to provide important insights into the pathogenesis and treatment of cardiovascular disease. The imaging of cell death in vitro is well established, and fluorescent annexin V (henceforth, annexin) and vital fluorochromes such as propidium-iodide are frequently used in vitro to image both the kinetics and nature of cell death. Moreover, in a landmark study, this dual fluorochrome approach was used to image cell death in the myocardium of mice in vivo using intravital microscopy. In the clinical setting, however, annexin-labeled probes have been used alone as single imaging agents. Though of significant value, the information provided solely by annexin imaging does not allow apoptosis and necrosis to be distinguished from each other when both are present. In addition, no translatable imaging strategy has been developed to specifically label necrotic cells, characterize the temporal evolution of necrosis, and image the clearance of necrotic debris from injured myocardium.

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To meet these needs, we describe the development and use of a novel multimodal DNA-binding gadolinium chelate (Gd-TO) to image necrotic cell death in vivo by MRI. Gd-TO consists of a gadolinium-chelate, similar to those used clinically, and a DNA-binding vital fluorochrome (TO-PRO 1). Like Gd-DTPA (gadopentetic acid, Magnevist, Schering, Berlin), which was the control probe used in the study, Gd-TO can be imaged in vivo 10 to 30 minutes after injection.
The synthesis of Gd-TO was as described. Briefly, the iodohexyl derivative of thiazole orange was reacted with ethyl 6-N,N-dimethylamino-hexanoate to afford a linker with a free carboxyl group for chelate attachment. \( \text{p-NH}_2\text{-Bn-DTPA} \) (Macrocyclics, Dallas, TX) was then coupled to the carboxyl group, followed by reaction with gadolinium to yield the gadolinium chelate reporter of Gd-TO. The binding of Gd-TO to DNA was examined by simulation and with in vitro assays. Models for the binding of TO-PRO 1 and Gd-TO to DNA were generated, based on the experimentally determined NMR structure of a TO bound to complementary DNA oligonucleotides. Briefly, the MOE 2007.09 docking suite (Chemical Computing Group, Montreal) was used to build 10 base pairs of ideal B-form DNA and energy minimized structures of TO-PRO 1 and Gd-TO (MMFF94X force field to a constant of 0.05 kcal/mol). Because of the predicted solvent exposure of the gadolinium-chelate group and uncertainty in the accuracy of models of this group generated by drug design software, only the linker and amide group of Gd-TO were considered. Initial models were generated by superimposing the coordinates for B-form DNA and TO-PRO 1 or Gd-TO onto the respective groups in 108D.pdb. Finally, the local interactions between the ligands (Gd-TO and TO-PRO 1) and the model DNA were optimized and minimized using the MOE LigX function. This approach is similar to that used previously on similar molecules.

The binding of Gd-TO to DNA and serum albumin was then studied in vitro. Gd-TO (35 \mu M/L) was incubated with calf thymus DNA (46 mg/mL, 1380 \mu M/L base), bovine serum albumin (40 mg/mL, 69 \mu M/L), or phosphate-buffered saline at 37°C for 40 minutes. The filtrate from this incubation was obtained by centrifugation through a 10-kDa cutoff Amicon Ultra filter (Millipore, UFC501024). The concentration of Gd-TO in the filtrate was then determined spectrophotometrically (OD510). A high concentration of Gd-TO in the filtrate indicated minimal binding, whereas a low concentration indicated strong binding.

### Experimental Protocol

The experimental scheme used is shown in Figure 1. MI was induced in 20 C57Bl6 mice by permanent ligation of the left coronary artery. Five of the infarcted mice were injected with Gd-DTPA (0.1 mmol/kg, ip) 24 to 48 hours after infarction (mean, 28.8±4.8 hours). The remaining 15 mice were injected with Gd-TO (0.1 mmol/kg, ip) at various time points after MI. T1-weighted MRI scans to detect the presence of gadolinium in the infarct were performed in all mice 2 to 3 hours after the injection of the imaging agent. This allowed the unbound fraction of both probes to be washed out before imaging and ensured that any gadolinium retention would reflect active binding.

MRI was performed on a 9.4-T horizontal bore magnet (BioSpec, Bruker, Billerica, MA) equipped with a 1500 mT/m gradient insert (Resonance Research Inc, Billerica, MA). Before T1-weighted imaging to detect the presence of gadolinium, gradient echo cines were acquired in the short axis of the left ventricle.
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notionally binding constant of Gd-TO to DNA, which was determined in the filtrate. DNA bound and retained Gd-TO, whereas BSA and the PBS control did not. **P<0.001.

using cardiorespiratory gating (SA Instruments, Stonybrook, NY) and the following settings: slice, 1 mm; FOV, 25×25 mm; matrix, 200×200; flip angle, 30°; 20 frames per R-R interval; TE, 1 ms; 4 averages. Slices showing wall motion abnormalities on the cine images were deemed to be infarcted and underwent further scanning to detect the presence of gadolinium with an ECG-gated inversion recovery (also known as a modified Look-Locker) sequence (Figure 1).

The ECG-gated inversion recovery recovery sequence consisted of a slice-selective adiabatic 180° inversion pulse, which was applied every 3 seconds. Signal along the longitudinal relaxation curve was sampled at multiple inversion times (TI), each defined by a multiple of the R-R interval (Figure 1). It should be noted that the T2 in the myocardium in mice at 9.4 T is approximately 20 ms and thus significantly shorter than the R-R interval (approximately 120 ms). A spoiled or unspoiled readout can thus be used without T2 contami-

tation of successive images along the inversion recovery curve. At each TI, 4 lines of k-space were acquired with a FISP sequence and the following parameters: FOV, 25×25 mm; slice, 1 mm; matrix, 160×160; flip angle, 20°; TR, 3.5 ms; TE, 1.38 ms; 4 averages. Maps of the longitudinal relaxation rate (\( R_1 \)) in the myocardium were constructed offline using Matlab (Mathworks, Natick, MA). Specif-
ically, the apparent longitudinal relaxation rate \( R_1^* \) was first ob-
ained by fitting the inversion recovery curve, \( S(t) = A \cdot Bc^{(-d_o\cdot R_1^*)} \), using a nonlinear least square algorithm. The \( R_1^* \) values were then obtained by modifying the apparent \( R_1^* \) as previously described, \( R_1^* = \frac{R_1}{(B/A)-1} \), for this sequence. The accumulation of Gd-TO or Gd-DTPA in the infarct was quantified by measuring \( R_1 \) in those portions of the myocardium showing wall motion abnormalities on cine MRI.

After MRI, 16 of 20 of the mice were immediately euthanized, and the hearts were harvested and sectioned for fluorescence microscopy. Fluorescence microscopy for Gd-TO was performed on an Olympus IX51 Inverted microscope with the following filters: excitation wavelength, 460 to 490 nm; emission wavelength, 510 to 550. The slides were then stained in situ with DAPI (Sigma, St Louis, MO) and imaged with the following filters: excitation wavelength, 330 to 385 nm; emission wavelength, 420 nm. Four of the 15 mice injected with Gd-TO (2 with infarcts <48 hours in duration and 2 with infarcts >72 hours in duration) were euthanized 24 hours after the Gd-TO injection (21 hours after the MRI) for inductively coupled plasma mass spectrometry (ICP-MS) of gado-
inium biodistribution. ICP-MS for gadolinium was performed in the blood, heart, lungs, brain, kidney, liver, spleen, bone, stomach, pancreas, and intestines. All samples were digested in 70% nitric acid overnight at 40°C. A Dysprosium solution was added as an internal standard to correct for any evaporative loss. Each digest was then diluted into a 5% nitric acid solution containing lutetium as an internal standard. Metal ions were assayed using an Agilent 7500 Series ICP-MS, and the gadolinium concentration was determined by comparing the Gd:Lu count ratio in each sample to a standard curve. Each digest was measured in duplicate. Gadolinium content in each organ was calculated as percentage injected dose (%ID) per organ and %ID per gram.

The uptake of Gd-TO by activated macrophages was also studied. Activated macrophages were obtained through peritoneal lavage in a mouse model of thioglycollate-induced peritonitis. The activated macrophages were exposed to 1 μmol/L of Gd-TO at 37°C for 15 minutes. A second group of activated macrophages was exposed to Gd-TO after their cell membranes had been permeabilized with a surfactant (Cytosix/Cytoperm Fixation/ Permeabilization kit, Becton-Dickinson, Franklin Lakes, NJ). Gd-TO uptake in the 2 groups of cells was assessed with flow cytometry (4 Laser LSR II, Becton-Dickinson). In addition, fluorescence microscopy for Gd-TO and DAPI in the 2 cell populations was performed as described above.

Statistical analysis of the data was performed with Prism (Graph-pad, La Jolla, CA). Comparisons between more than 2 groups were performed with an ANOVA test and a Tukey posttest comparison. Values throughout the paper are expressed as mean±SEM, and a probability value of <0.05 was required to meet significance. All animal studies described in the report were conducted in accordance with the policies for research animal use at our institution and with the approval of the subcommittee for research animal use at our institution.

Results

The essential features of Gd-TO, its structure, binding to DNA, and lack of albumin binding, are shown in Figure 2. The design of Gd-TO (Figure 2) is based on the precursor structure TO-PRO-1 (Figure 2A and 2B), which is a membrane impermeable vital dye.6,10 The quaternary ni-
trogen on TO-PRO 1 provides a permanent positive charge that likely prevents its entry into healthy (vital) cells. The design of Gd-TO (Figure 2C) preserves the rings of TO, and the quaternary nitrogen of TO-PRO 1, while adding a 6-carbon carboxyl group terminated linker (red) and chelated Gd reporter (green). The results of our simulation studies revealed that the attachment of a Gd chelate to TO-PRO 1 did not change its binding to DNA. The rings of TO-PRO 1 (Figure 2D) and Gd-TO (Figure 2E) intercalated similarly into double stranded DNA (arrows), whereas their quaternary amines formed electrostatic bonds (dotted rectangles) with the phosphate groups of DNA. Our model indicates that when Gd-TO binds DNA, its 6-carbon linker swings out from the double helix and is unlikely to affect binding.

In vitro binding assays confirmed the affinity of Gd-TO for DNA. Incubation of Gd-TO (35 μmol/L) with DNA (1360 μmol/L base) resulted in profound retention of the agent and the absence of Gd-TO in the filtrate (Figure 2F). In contrast, both albumin (69 μmol/L) and phosphate-buffered saline failed to bind Gd-TO, yielding a filtrate that contained the full concentration of Gd-TO. The failure of Gd-TO to bind albumin is noteworthy given the affinity of many other fluorochromes (eg, indocyanine green, bromocresol green, cibacron blue) for serum albumin.

Gadolinium-TO (Gd-TO) uptake in an infarcted mouse, imaged 9 hours after LCA ligation. Three frames of an ECG-gated inversion recovery sequence are shown, each separated by 2 R-R intervals (heartbeats). A, At the inversion time (TI) of R-R (n), the signal in the infarcted myocardium has reached the null point (NP) and appears dark. The signals in the blood pool (BP) and septum (white arrow) have slower relaxation rates and have not yet reached the NP. B, At R-R (n+2), the signals in the BP and septum are nulled, whereas the infarct has passed through the NP and appears bright (yellow arrows). C, At R-R(n+4), all signals have passed through the NP. However, the high content of Gd-TO in the infarct has produced a greater degree of longitudinal relaxation and the infarct thus appears hyperintense.

ANOVA was performed with the mice divided into the following 3 groups (Figure 5C): those injected with Gd-DTPA within 24 to 48 hours of infarction (mean time, 28.8±4.8 hours), those injected with Gd-TO less than 48 hours from the onset of infarction, and those injected with Gd-TO 72 hours or more after infarction. Significant differences were seen in the R1 of those mice injected with Gd-TO less than 48 hours from infarction and those injected with Gd-TO 72 hours or more after infarction.
Likewise, significant differences were present in the mice injected with Gd-TO within 48 hours of infarction and those injected within 24 to 48 hours of infarction with Gd-DTPA (1.24±0.08 versus 0.76±0.02 s⁻¹, P<0.001).

No evidence of Gd-TO accumulation was seen either by MRI or fluorescence microscopy in noninfarcted myocardium. However, in those mice imaged within 48 hours of infarction, large amounts of Gd-TO were seen within the infarct. Patchy uptake of Gd-TO was also seen in these mice in the infarct border zone, consistent with the presence of normal and necrotic cells in this region. An excellent degree of colocalization was seen between Gd-TO and a DAPI (nuclear) costain, confirming that Gd-TO was bound to the nuclei of necrotic cells. These findings are well demonstrated in the fluorescence microscopy images of a mouse injected with Gd-TO and imaged 18 hours after MI (Figure 6A through 6D). Flow cytometry of the activated macrophages (Figure 6E) revealed that Gd-TO was taken up by these cells only once their cell membranes had been permeabilized. Gd-TO accumulation in the permeabilized macrophages colocalized very strongly with DAPI staining (Figure 6F through 6H). Gd content, determined by ICP-MS in 4 mice 24 hours after the injection of Gd-TO, is shown in the Table. Overall, less than 4% of the injected Gd remained in the body 24 hours after injection.

**Discussion**

The central role of cell death in cardiovascular disease makes the detailed understanding of this process imperative. We present here a novel DNA-binding agent, Gd-TO, to image necrotic cell death in vivo by MRI. The specificity of Gd-TO for acute necrotic cell death is particularly evident in Figure 5, where the uptake of Gd-TO is shown to be significantly different from that of Gd-DTPA in myocardium with infarction times of less than 48 hours compared to those with times of greater than 72 hours (P<0.001). Similarly, significant differences (P<0.001) in myocardial R1 were seen between mice with acute infarcts (<48 hours) injected with Gd-TO and those injected with Gd-DTPA. The accumulation of Gd-TO thus specifically identifies acutely necrotic cells.
demonstrated in a mouse model of MI. We show that the agent has the unique ability to image both the mechanism and chronicity of cell death through a novel and well-elucidated molecular mechanism. Although we focus in the current study on a mouse model of MI, the utility of the agent is extremely broad and it can be used to image necrotic cell death in any cardiovascular condition. A versatile, well-characterized, and effective platform for the imaging of acute necrotic cell death is thus presented.

The kinetics and mechanisms of cell death in the myocardium remain incompletely understood. Moreover, the response of serological biomarkers such as troponin to injury within a solid organ can be highly delayed and nonlinear. In contrast, the use of a vital imaging agent such as Gd-TO provides a direct and spatially resolved readout of cell death at the local level. Gd-TO uptake in this study was present within 2 hours of MI and peaked between 9 to 18 hours of injury. This suggests that cell rupture and significant changes in membrane permeability begin within 1 to 2 hours of infarction. Full disintegration and rupture of the cell, however, takes many more hours to occur and maximal uptake of Gd-TO thus occurred 9 to 18 hours after infarction. This time course is consistent with previous histological data using an antimyosin antibody. Notwithstanding differences in human and murine pathophysiology, the kinetics of Gd-TO accumulation provide valuable insights into the kinetics of serological biomarkers such as troponin. Troponin elevation in patients with acute coronary syndromes is frequently detected only 6 to 12 hours...
after injury, 14 well after the initial uptake of Gd-TO in this study. This suggests that the release of troponin into the extracellular space is a late event in cardiomyocyte necrosis and that elevations in serum troponin will be seen only once profound disintegration of the cardiomyocyte membrane has occurred.

The response of the inflammatory system to the release of cellular DNA has been extensively studied. 16, 17 Free DNA in its own right serves as a danger signal, stimulating an inflammatory response. 16 DNA also interacts with toll-like receptors on monocytes, 18 promoting phagocytosis. In addition, several opsonins that bind DNA and promote its phagocytosis have been identified including C1q, 19 mannose-binding lectin, 20 ficolin-2 and 3, 21 properlydin, 22 and histidine-rich glycoprotein. 23 These and other mechanisms result in a robust monocyte infiltrate within the infarct, which in mice reaches full threshold approximately 24 hours after injury. 24, 25 The absence of Gd-TO uptake 72 to 96 hours after MI suggests that the removal of necrotic cell debris by infiltrating monocytes is completed within 2 to 3 days, consistent with prior histological studies. 24 Interestingly, this time point corresponds to the transition of the monocytes infiltrating infarcted myocardium from highly degradative lys6C-high monocytes to more reparative lys6C-low monocytes. 25

Antibodies targeted to specific antigens, such as myosin, 26 can be used to image membrane integrity. However, antibodies are far too large for renal elimination and frequently show high nonspecific hepatic accumulation. The simultaneous use of radiolabeled annexin and anti-myosin antibodies, 27, 28 although of value, is thus significantly limited by these factors. In contrast, the molecular weights of TO and the complete Gd-TO molecule are 305 Da and 1110 Da, respectively. The physical properties of Gd-TO (small size, water solubility, lack of albumin binding) probably contribute to its rapid clearance from the blood and its low tissue retention at 24 hours (Table), a prerequisite for the clinical translation of any gadolinium-based agent. Imaging of Gd-TO uptake was performed 2 to 3 hours after injection in this proof-of-principle study. However, images acquired in our study 1 hour after injection suggest that this time point would also be highly suitable for the imaging of Gd-TO.

The development of T2-weighted MRI sequences to image tissue edema has been a major advance. 29, 30 However, T2 hyperintensity is a nonspecific signature and cannot differentiate acute (less than 72 hours) from subacute (less than 6 weeks) injury. The use of serological biomarkers such as troponin is frequently helpful, but not definitive, in these situations. Regions of acute and subacute injury, for instance, can frequently coexist and cannot be easily distinguished from each other using T2-weighted MRI and serological biomarkers. The availability of a single definitive imaging test to differentiate acute from subacute injury would thus be a valuable advance. Moreover, the use of Gd-TO to study the mechanism, kinetics, and immune response to cell death has the potential to yield valuable insights into myocardial injury and result in improved therapeutic strategies.

Delayed (late) enhancement imaging of extracellular chelates in the myocardium is based on changes in the extracellular volume of distribution of the chelate and is a nonspecific finding seen in both acute and chronic injury. 31, 32 Gadolinium chelates such as gadoporphyrin, which preferentially accumulate in necrotic tissue, have been developed. 33, 34 However, the mechanism of uptake of these agents is completely nonspecific and is probably related to binding to a variety of proteins and connective tissue elements. In contrast, the uptake of Gd-TO occurs via a specific and well-elucidated molecular mechanism and is thus able to provide novel insights into the kinetics of cell death and clearance in infarcted myocardium.

Molecular MRI of apoptosis in vivo has been performed with several constructs, most notably the superparamagnetic nanoparticle AnxCLIO-Cy5.5. In an initial proof-of-principle study in the heart, this agent was used alone and was thus not able to distinguish apoptosis from necrosis. 35 In a subsequent study, delayed enhancement of gadolinium (Gd-DTPA) was used in conjunction with AnxCLIO-Cy5.5 to attempt to resolve the various forms of cell death in the myocardium. 36 While of value, the nonspecific nature and limitations of delayed enhancement imaging have been discussed above. The availability of AnxCLIO-Cy5.5 and Gd-TO now makes it possible to replicate, in the in vivo setting, the robust dual fluorochrome approach used during intravital microscopy and flow cytometry.

In conclusion, the work presented here is, to the best of our knowledge, the first demonstration of molecular MRI with a multimodal vital imaging agent. We show that Gd-TO is able to image the mechanism of cell death as well as the evolution and clearance of necrotic cells in acute ischemia. In addition, we show that inflammatory cells do not take up Gd-TO, making the agent suitable for imaging necrotic cell death in highly inflammatory milieus such as infarcted myocardium, myocarditis, and transplant rejection. Gd-TO does not require the synthesis of new biological materials and is well suited to synthesis at the scale needed for large-animal and human studies. The

Table. Inductively Coupled Plasma Mass Spectrometry 24 Hours After the Injection of Gd-TO

| Organ       | %ID per Gram | %ID per Organ |
|-------------|--------------|---------------|
| Heart*      | 0.0102±0.0037 | 0.0012±0.0004 |
| Spleen      | 0.115±0.0339  | 0.0091±0.0027 |
| Stomach     | 0.0916±0.0268 | 0.0291±0.0085 |
| Liver       | 2.74±0.3809   | 2.63±0.3655   |
| Kidneys     | 0.784±0.1739  | 0.206±0.0457  |
| Intestines  | 0.493±0.3248  | 0.774±0.5101  |
| Lungs       | 0.0299±0.0077 | 0.0040±0.0001 |
| Bone        | 0.0409±0.0047 | 0.0819±0.0094 |
| Brain       | <0.006       | <0.002        |
| Blood       | <0.02        | <0.03         |
| Pancreas    | <0.005       | <0.0008       |

ID indicates injected dose; Gd-TO, DNA-binding gadolinium chelate.

*Half the animals studied with inductively coupled plasma mass spectrometry had infarcts >72 hours in duration at the time of Gd-TO injection.
structure, binding, and mechanism of uptake of Gd-TO are all well understood, and it is well eliminated. The potential of Gd-TO, or a radioactive TO analog, to undergo successful clinical translation is thus high.

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Disclosures

None.

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Necrotic cell death can be detected in vitro with DNA-binding vital fluorochromes such as propidium-iodide. We present gadolinium-TO (Gd-TO), a DNA-binding gadolinium chelate for the specific imaging of necrotic cell death by MRI in vivo. Gd-TO consists of a Gd chelate conjugated to the DNA-binding vital dye TOPRO-1. We show, in a mouse model of myocardial infarction (MI), that Gd-TO robustly images acute necrotic cell death in vivo. Although uptake of Gd-TO was seen within 2 hours of infarction, peak uptake occurred 9 to 18 hours after MI. This pattern parallels the release of troponin by cardiomyocytes and suggests that cardiomyocyte rupture peaks many hours after the onset of myocardial ischemia. No uptake of Gd-TO was seen in mice injected with the agent more than 72 hours after MI. This suggests that necrotic cells are rapidly and efficiently cleared from the myocardium within 72 hours of injury. Gd-TO provides a readout that is specific for acute (<72 hours) necrotic cell death and can be used to assess the kinetics, mechanism (apoptosis versus necrosis), and chronicity of cell death in vivo. Current techniques such as delayed enhancement and T2-weighted MR imaging, while of major value, are not specific for cell death and may not be able to differentiate acute (<72 hours) from subacute (<30 days) injury. Gd-TO thus has the potential to become a useful clinical tool and play an important role in the diagnosis and characterization of cell death.