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Multimodality imaging demonstrates trafficking of liposomes preferentially to ischemic myocardium

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Abstract

Introduction—Nanoparticles may serve as a promising means to deliver novel therapeutics to the myocardium following myocardial infarction. We sought to determine whether a lipid-based liposomal nanoparticles can be shown through different imaging modalities to specifically target injured myocardium following intravenous injection in an ischemia-reperfusion murine myocardial infarction model.

Methods—Mice underwent ischemia-reperfusion surgery and then either received tail-vein injection with gadolinium- and fluorescent-labeled liposomes or no injection (control). The hearts were harvested 24 hours later and underwent T1 and T2-weighted ex vivo imaging using a 7 Tesla Bruker magnet. The hearts were then sectioned for immunohistochemistry and optical fluorescent imaging.

Results—The mean size of the liposomes was 100 nm. T1-weighted signal intensity was significantly increased in the ischemic vs the non-ischemic myocardium for mice that received liposomes compared with control. Optical imaging demonstrated significant fluorescence within the infarct area for the liposome group compared with control ($163 \pm 31\%$ vs $13 \pm 14\%$, $p=0.001$) and fluorescent microscopy confirmed the presence of liposomes within the ischemic myocardium.

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Conclusions—Liposomes traffic to the heart and preferentially home to regions of myocardial injury, enabling improved diagnosis of myocardial injury and could serve as a vehicle for drug delivery.

Keywords

Myocardial infarction; Imaging; Nanoparticle; Liposome; Ischemia

Introduction

The past decade has witnessed major improvements in outcomes following myocardial infarction (MI) (1). These may be attributed to improvements in revascularization with percutaneous coronary intervention, and to more aggressive use of medical therapies including anti-platelet agents, anticoagulation, beta-blockers, inhibitors of the renin-angiotensin-aldosterone system, and statin therapy. However, despite enhanced outcomes, the development of heart failure (HF) following AMI remains high and is associated with significant morbidity and mortality(1). It therefore seems important to consider additional strategies that could lead to further improvement in long-term outcomes of patients with MI.

The use of liposomes as drug delivery platforms is well established in oncology. An example of this is the utilization of pegylated liposomes for the delivery of chemotherapeutics(2). Liposomes provide the ability to increase the duration of circulation for agents with short half-lives and may provide a means to enter the infarcted myocardium through "leaky" endothelium(3). Lipid-based nanoparticles have been utilized to image myocardial infarction(4) and can be modified to target specific molecules in the ischemic or infarcted myocardium(5,6). Studies have been performed using liposomes carrying drugs capable of reducing inflammation(7), stimulating repair(8), increasing angiogenesis(9), and augmenting cardiac function(10). In addition to therapeutic potential, lipid-based nanoparticles have been utilized to image myocardial infarction(4) and can be modified to target specific molecules in the ischemic or infarcted myocardium(5,6).

In the present investigation we sought to determine, using different imaging modalities, whether liposomes specifically target injured myocardium following intravenous injection in an ischemia-reperfusion murine myocardial infarction model. It is our belief that if targeting is relatively specific and robust, then such a delivery platform could prove of value for delivery either imaging agents to identify regions of myocardial infarction, or therapeutic agents to reduce the extent of myocardial injury.

Methods

Liposome Preparation

Liposomes were made from the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), the fluorescently-labeled phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-7-nitro-2-1,3-benzoxadiazol-4-yl (DPPE-NBD), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide-(poly(ethylene glycol))2000] (DSPE-PEG2000-Mal), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-

[methoxy(polyethylene glycol)-2000] (DPSE-PEG2000), and the aliphatic Gd complex Gd-diethylenetriamine pentaacetate-bis(stearylamide) (Gd-DTPA-BSA)) in a molar ratio of 78:2:1:12:7. The lipid mixture was dissolved in a 1:1 chloroform/methanol solution (5 ml) and evaporated under nitrogen flux yielding a thin film that was then rehydrated. Thereafter, the lipid film was heated and sonicated twice for 15 min at 70W at 90% duty cycle. The DSPE-PEG2000-Mal can be utilized to attach the resulting liposome to a monoclonal antibody to target specific molecule. Dynamic light scattering was performed on a Malvern instrument (Zetasizer, Nano-S) to determine the hydrodynamic diameter of a suspension of liposomes. The number of gadolinium ions per liposome was determined by inductively coupled plasma mass spectroscopy (Maxxam Analytics, Burnaby, British Columbia, Canada) and this data was used to determine the number of NBD molecules per liposome.

Ischemia Reperfusion Surgery and Liposome Injection

Animal care and procedures were carried out in accordance with the guide for the care and use of laboratory animals and our protocol was approved by the MedStar Health Research Institute Institutional Animal Care and Use Committee. All animal procedures were carried out at the Medstar Heart and Vascular Institute. Adult chow-fed, male CD1 mice at 8 to 10 weeks of age were anesthetized with pentobarbital sodium at 70mg/kg via intraperitoneal injection with additional doses at 10 mg/kg as needed. The mice underwent endotracheal intubation and were ventilated w/ room air at a rate of 170 to 220 breaths per minute with a tidal volume 0.27 mL using a rodent ventilator (Harvard Apparatus). Left lateral thoracotomy was performed under sterile fashion and the left anterior descending (LAD) coronary artery was temporarily ligated just below the left atrial appendage with a 7-0 silk suture (Ethicon) using a loose knot tied over a small piece of Intramedic polyethylene PE 50 tubing (Becton, Dickinson and Company) to temporarily occlude blood flow and cause obvious blanching of the anterior left ventricular myocardium. Following 45 minutes of ischemia, the tubing was removed to re-establish blood flow and reperfusion was verified by reactive hyperemia. The suture was left in place in the myocardium, the ribs and skin were closed with a 5-0 Nylon suture (Ethicon), the animal recovered from anesthesia, and received standard post-operative pain control. The following day, animals either received a tail vein injection of 200 μ L of liposomes or no injection. Approximately 24 hours later, mice received a lethal dose of pentobarbital, blood was aspirated from the right ventricle, and the heart and organs were harvested from each animal. The hearts were placed on a plastic rod to maintain orientation and placed in Fomblin solution (perfluoropolyether; Solvay Solexis) for ex vivo imaging.

Magnetic Resonance Imaging

Ex vivo magnetic resonance (MR) imaging was performed using a 7 Tesla Bruker scanner (Billerica, Mass) with 29 G/cm gradients. A T1-weighted 3D gradient echo imaging sequence (TR/TE, 100/3.2 ms; flip angle, 60°; 3 averages) was used. The field of view was generally 2.5×1×1 cm, and the matrix was 128×64×64, to yield a voxel size of 195×156×156 μ m. T2-weighted spin echo images (TR/TE, 1500/12 ms; flip angle, 180°) were obtained for each heart through a representative section of the infarct and corresponding T1-weighted images were obtained to perform T1 signal intensity analysis. An independent cardiologist (ROE) blinded to treatment allocation (liposomes vs control)

selected regions of interest (ROI) within the infarct/ischemic region supplied by the LAD artery and a corresponding ROI from the inferoseptal wall (non-ischemic region) to generate average pixel intensity from T1-weighted images for each heart using ImageJ software (NIH, Bethesda, Maryland).

Immunohistochemistry

Following MR imaging, the hearts were placed in OCT embedding medium for frozen tissue specimens (Tissue-Tek) to preserve the orientation of the LAD territory. Five micron thick sagittal or axial sections of the heart were then cut to keep a short-axis orientation using a Thermo Shandon Cryotome (Pittsburg, PA). Following creating slides for frozen sectioning, a 1.5 mm thick short-axis section was cut using 3 slots of the 0.5 mm Zivic heart slicer (Zivic Instruments) to be used for optical imaging. Slides were then fixed and permeablized in acetone at -20°C , washed in phosphate buffered saline (PBS), incubated in 10% BSA in PBS for 1 hour, washed in PBS, incubated with Rphycoerythrin (PE)-labeled anti-mouse monoclonal antibody against CD11b (BioLegend, San Diego, CA) or PE-labeled anti-mouse monoclonal antibody targeting CD45 (BioLegend, San Diego, CA) at 1:100 in 1% BSA in PBS for 1 hour at room temperature, washed in PBS, and treated with DAPI mounting media. Fluorescent microscopy was performed using a Zeiss Axioimager microscope and multichannel black and white camera equipped with fluorescence filters (Carl Zeiss, Oberkochen, Germany).

Optical Imaging

Ex vivo optical fluorescent imaging of the 1.5 mm thick axial or short-axis heart sections was performed with a Xenogen IVIS 100 in vivo imaging system (Caliper Life Sciences) using the GFP channel (excitation 445-490 nm/ emission 515-575 nm). and comparison between groups. Fluorescent imaging of all heart sections was performed at the same time by obtaining a single fluorescent image of all heart sections in close proximity to enable uniform measurement of fluorescence, minimize variability, and enable quantitative fluorescent comparison using the same settings. ROIs were again selected of the whole heart section, from the LAD territory, and from the non-ischemic region to obtain the mean level of fluorescence for each ROI.

Statistics

Mean signal intensity values obtained from T1-weighted MR images and fluorescence imaging for the control and liposome groups were compared using Student's T test (Prism 6 software, San Diego, CA). Statistical significance was defined as a p-value <0.05 .

Results

The liposomes had a mean diameter of 100 nm with a narrow size distribution (range of 85 to 115 nm). Inductively coupled plasma mass spectroscopy demonstrated an average of 27,000 gadolinium DTPA molecules per liposome, and an average of 6,700 NBD molecules per liposome. Figure 1 provides an illustration of our liposome and its composition. Following ischemia-reperfusion surgery, all 7 mice randomly allocated to the liposome group tolerated the 200 μL tail-vein injection of liposomes without any adverse effect. The

control group was composed of an additional 7 mice that underwent ischemia-reperfusion surgery that did not receive tail-vein injection.

Magnetic Resonance Imaging

Hearts were harvested 24 hours following either tail-vein injection of liposomes or no injection (control), which was approximately 48 hours after MI. Representative ex vivo T1- and T2-weighted MR axial or short-axis images were obtained for each heart. The T2-weighted images confirmed the presence of myocardial edema (bright signal in the LAD territory) as a result of ischemia in the all hearts, but demonstrated variability in the amount of myocardial edema. It is also possible that some of the increased enhancement in the T2-weighted images could represent enhancement from the liposomes in the liposome group. Additionally, the ex vivo imaging with the 7 Tesla magnet enabled excellent myocardial characterization as can be seen in Figure 2 where the high resolution imaging enabled identification of intramyocardial hemorrhage or microvascular obstruction (dark regions of decreased signal) along with myocardial edema on T2-weighted imaging.

Hearts of mice that received liposomes had a clear increase in signal intensity in T1-weighted images in the LAD territory compared with the non-ischemic region, while there was no appreciable increase in signal intensity in the LAD territory of the control hearts (Figure 2). In the T1-weighted cross-sectional images, the percent increase in the average signal intensity of the LAD territory relative to the non-ischemic territory was significantly greater in the hearts of mice that received liposomes compared with the hearts of mice that had not received liposomes ($41 \pm 10\%$ vs $9 \pm 2\%$, $p < 0.01$, Figure 3A).

We further demonstrated that the percent increase in maximal signal intensity in the LAD territory relative to the non-ischemic territory of the T1-weighted cross-sectional images was significantly greater in hearts derived from mice receiving liposomes compared with the hearts of mice not receiving liposomes ($83 \pm 9\%$ vs $17 \pm 5\%$, $p < 0.0001$, Figure 3B).

When comparing the signal intensity in the non-ischemic territory for T1-weighted short-axis images, there was no increase in signal intensity in the non-ischemic territory from mice that received liposomes compared with the control mice (Figure 3C). This suggests that the liposomes are not significantly retained in non-ischemic myocardium and therefore do not impact the signal intensity on T1-weighted images.

Fluorescent Optical Imaging

Following frozen sectioning, a 1.5 mm thick short-axis or axial section was cut for each heart and the heart sections underwent fluorescent optical imaging with maintenance of the LAD orientation. The heart sections from mice that received tail-vein injection of liposomes demonstrated significant fluorescence in the LAD territory while the heart sections from the control mice only showed background fluorescence (Figure 4A). The heart sections from the mice that received liposomes demonstrated a 50% increase in total fluorescence compared with the control heart sections when the total fluorescence was normalized to the control hearts (1.50 ± 0.09 vs 1.00 ± 0.06 , $p = 0.0007$, Figure 4B). Furthermore, the increase in fluorescence in the LAD territory relative to the non-ischemic territory for the heart sections was significantly greater for mice that received liposomes compared with the control mice

($163 \pm 31\%$ vs $13 \pm 14\%$, $p=0.0008$, Figure 4C). Importantly, when comparing the fluorescence in the non-ischemic territory for the heart sections, there was no increase in fluorescence for the heart sections from mice that received liposomes compared with the control mice (Figure 4D). This confirms that the liposomes are trafficking to ischemic myocardium and are not significantly retained in non-ischemic myocardium.

Immunohistochemistry

Following frozen sectioning, slides were stained for CD45 (red), a cell surface molecule present on all nucleated hematopoietic cells(11), and CD11b (red), an integrin present predominantly on mononuclear cells(12). The mounting media contained DAPI which stains the nuclei blue. In the LAD territory, fluorescent microscopy demonstrated the presence of intense green fluorescence consistent with NBD-labeled liposomes and the presence of cells staining positive for both CD11b and CD45 (Figure 5). However, the control hearts demonstrated only the green background autofluorescence of the myocardium while both CD45 and CD11b-positive cells are present.

Figure 5 also demonstrates negligible intense green fluorescence in the non-ischemic myocardium along with fewer leukocytes, indicating liposome retention is predominantly limited to the region of myocardial ischemia. With higher magnification (10X and 40X), it appears that intense green fluorescence is intracellular, given the clear cellular structures and presence of nuclei (Figure 6).

Discussion

The results of our study definitively demonstrate that following MI, liposomes traffic to the heart after iv injection and preferentially home to regions of myocardial injury. We were able to reach this conclusion by successfully generating liposomes capable of serving as MR and fluorescent optical imaging agents. Thus, our liposomes clearly increased the MR T1 signal intensity within the LAD territory following ischemia-reperfusion compared with control animals, and also resulted in the appearance of fluorescence in the LAD territory. Importantly, while signal intensity or fluorescence increased in the LAD territory vs. non-ischemic region of the LV following ischemia-reperfusion after injection of liposomes, there was no difference in signal intensity in the non-ischemic territory of MI hearts vs. hearts not subjected to MI. This further proves that liposome uptake is largely limited to the regions of myocardial ischemia. This implies the potential for liposomal nanoparticles to serve as a diagnostic imaging agent for identification of myocardial injury. Ex vivo fluorescence imaging confirmed this, demonstrating evidence of liposome retention within the region of myocardial ischemia. Finally, fluorescent microscopy demonstrated liposomes were largely confined to the ischemic region of myocardium.

Our study's findings are consistent with those of Geelen and colleagues who demonstrated that liposomes can identify regions of myocardial injury with MR imaging and fluorescent microscopy at 24 hours following injection of liposomes(4). However, in our study we utilized ex vivo MR imaging with a 7 Tesla magnet which yielded impressive image resolution and even enabled characterization of the myocardial infarction. Interestingly, they demonstrated that liposomes are not taken up by myocardial at 3 hours following ischemia-

reperfusion injury and injection of liposomes; rather, significant uptake occurred by 24 hours. This suggests time is required for leaky endothelium to develop. In this regard, we performed ex vivo MR imaging 24 hours following liposome injection but 48 hours following ischemia-reperfusion injury. As expected from the results of Geelen and colleagues, this later time of injection of liposomes resulted in significant uptake of the liposomes by the ischemic tissue.

Of mechanistic importance, Dauber and coworkers demonstrated that endothelial intercellular gaps develop in blood vessels supplying injured myocardium in the setting of ischemia-reperfusion(13). These “leaky” vessels enable the entry of macromolecules, including liposomes, into the ischemic myocardium(3,14). This “leaky vessel” concept is supported by our study in which we demonstrated that injected liposomes are largely present in ischemic territory and are not significantly increased in non-ischemic territory.

Since liposomes have the ability to incorporate both molecular imaging agents and novel therapeutic agents, the leaky vasculature present in ischemic tissue would enable retention of liposomes loaded with therapeutics within the ischemic myocardium. The additional theoretical advantage of using liposomes as a therapeutic delivery platform is that the therapeutic agent would continue to be deposited over an extended time, given the long half-life of liposomes in the circulation.

While many studies have employed liposomes targeted to specific molecules within the infarcted myocardium(7–9), our study suggests that the appearance of leaky vessels in ischemic tissue will provide a sufficient “homing” mechanism—largely limiting the localization of the liposomes to the ischemic tissue such that further targeting with antibodies or specific molecules may not be necessary for adequate liposomal targeting and retention. While targeting the nanoparticle to a component of the myocardium may improve retention compared with non-targeted nanoparticles(15), the conjugation of such molecules or antibodies may increase cost of synthesis without increasing therapeutic effect. Further studies are necessary to determine whether loading nontargeted liposomes with therapeutic agents will provide sufficient local delivery of the therapeutic agent (relying on the natural targeting provided by ischemia-induced leaky vessels) to produce a biologically meaningful beneficial effect in ischemic tissue, or whether incremental benefit will accrue by creation of targeted nanoparticles.

A limitation of this study is the lack of data regarding infarct size for each short-axis slice by gross histopathological determination with triphenyltetrazolium chloride (TTC) staining. This would enable us to correlate the size of myocardial infarction with the degree of signal intensity on MR and fluorescent imaging. Unfortunately, the duration of time required for ex vivo imaging could introduce variability in adequacy of TTC staining as longer durations of time lead to tissue necrosis and poor or inaccurate TTC staining. Additionally, the amount of tissue required for ex vivo fluorescent optical imaging and fluorescent microscopy did not leave adequate tissue for assessment of infarct size by TTC from short-axis sections. In future studies, we hope to determine whether the degree of infarct size directly correlates with degree of liposome retention. Finally, further studies such as flow cytometry may help identify which cells are taking up the liposomes as seen in Figure 6.

In conclusion, we demonstrated that liposomes, labeled with gadolinium and NBD, are preferentially taken up by ischemic myocardium and provide multi-modal imaging of myocardial infarction. These nanoparticles may therefore serve as a platform to not only image ischemic myocardium but to deliver novel therapeutic agents following MI.

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Figure 1.
Cartoon illustrating the chemical composition of the liposomes utilized for these experiments.

Figure 2.

Ex vivo MR images were obtained with the heart suspended on plastic rods passing through the left ventricular cavity and out the left ventricular apex. In the long-axis T1-weighted images (A), the apex of the heart is oriented toward to the top of the figure and the base of the heart is toward the bottom of the figure. All hearts were from mice that underwent ischemia-reperfusion surgery: 3 hearts from mice that received liposomes are on the top and the 3 control hearts on the bottom. The LAD territory is located on the right side and the inferior wall of the heart is on the left of the plastic rod seen in the middle of the heart.

Increased signal intensity (bright area) can be seen in the LAD territory of the hearts of liposome recipients while there was no increase in signal intensity in the control hearts. In the short-axis images (B), 2 representative hearts are shown for the liposome recipients on the left and 2 control hearts are shown on the right. Each heart shows a representative T1-weighted image (left) and a T2-weighted image (right). The LAD territory is located toward the top of the figure, the septal wall is to the left, the lateral wall is toward the right, and the inferior wall is on the bottom. T2-weighted images reveal myocardial edema (bright area) in the LAD territory consistent with myocardial ischemia in the representative hearts. There is even evidence of intramyocardial hemorrhage or microvascular obstruction (arrow) in the T2 imaging in the LAD territory. Increased signal intensity (bright area) in the LAD territory is present in the T1-weighted images of the hearts from mice that had received liposomes, while no increase in signal intensity was observed for control hearts.

Figure 3.

(A) Graph comparing the percent increase in the *mean* signal intensity of the ischemic (LAD) territory relative to the non-ischemic (inferoseptal) territory for the liposome group vs the control group from the T1-weighted images. (B) Graph comparing the percent increase in the *maximal* signal intensity of the LAD territory relative to the non-ischemic territory for the liposome group vs the control group. (C) Graph comparing the signal intensity in the non-ischemic territory for the liposome group and the control group which is presented as values relative to the control mean.

Figure 4.

Fluorescent optical images of short-axis sections through the area of ischemia-reperfusion injury in Figure 4A demonstrate the presence of fluorescent liposomes in the LAD territory in the two examples from the liposome group (left) while there is minimal fluorescence in the two control group examples (right). A single fluorescent image was obtained for all heart sections in close proximity to enable uniform measurement of fluorescence and comparison between groups. The anterior wall (supplied by the LAD) is oriented to the top, the septal wall is on the left, the lateral wall is on the right, and the inferior wall is on the bottom. Figure 4B compares the total fluorescence of short-axis images for the liposome group compared with the control group normalized to the mean fluorescence of the control group. Figure 4C compares the percent increase in the mean fluorescence of the LAD territory relative to the non-ischemic territory for the liposome group vs the control group. Figure 4D compares the fluorescence in the non-ischemic territory for the liposome group and the control group, which is presented as values relative to the control mean.

Figure 5.

Intense green fluorescence confirms NBD-labeled liposomes accumulated in the infarct zone but were not present in the control hearts to any significant degree. Fluorescent microscopy images were obtained at low magnification with RGB merged channels with DAPI staining blue for the nuclei in all images, intense green fluorescence (NBD-labeled liposomes), background green fluorescence consistent with autofluorescence of the myocardium, and red staining for CD11b (top two images) and CD45 (bottom two images). The two images to the left are from the liposome group and demonstrate intense green fluorescence in the LAD territory while there is no intense green fluorescence in the LAD territory of the control group to the right. The arrow in the top left image points to an area on the border of the LAD territory showing minimal intense green fluorescence suggesting minimal liposome retention in non-ischemic myocardium. CD11b-positive cells can be seen in the LAD territory of both top images and CD45-positive cells can be seen in the LAD territory of both bottom images.

Figure 6.

Fluorescent microscopy images at 10X (left) and 40X (right) magnification for the liposome group with RGB merged channels with DAPI staining blue for the nuclei in all images, intense green fluorescence (NBD-labeled liposomes), background green fluorescence consistent with autofluorescence of the myocardium, and red staining for CD45 (bottom left only) and CD11b. The images suggest intracellular location of the intense green fluorescence (arrows).