Closed-chest cell injections into mouse myocardium guided by high-resolution echocardiography


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THE LAST SEVERAL YEARS have witnessed growing enthusiasm about the therapeutic potential of cardiac gene therapy and the implantation of putative adult stem cells (12). Numerous investigators (7, 11, 17, 27) have attempted to deliver these potential stem cells or angiogenesis-promoting genes to ischemic hearts by direct injection into the myocardium in an attempt to either cause myocardial regeneration or stimulate new blood vessel growth. However, these approaches have been subject to controversy. For example, the published data resulting from bone marrow cell injections vary from demonstrating a significant contribution to cardiac tissue to showing minimal, if any, contribution (3, 13, 17). Despite these contradictions, clinical trials have begun in several countries, showing initial results that are promising but require more investigation to be conclusive (2, 24–26, 29). Experimentation with mice and rats remains critical to understanding whether direct intramyocardial delivery of these cells can improve cardiac function after myocardial infarction and to understanding the role that these cells may play in cardiac functional improvement.

Rats have frequently been used because of their larger size; however, the mouse model offers more scientific options with the availability of inbred, genetically marked, and functionally transgenic strains. If it were not for its smaller size, the mouse model would clearly be the system of choice. Currently, most investigators ligate the mouse coronary artery to induce myocardial infarction by an open-chest approach and then inject cells directly into the heart immediately or within hours of the infarction. This approach has two problems. First, even a 30-gauge needle bevel (the smallest in common use) is comparable in size to the thickness of the mouse myocardial wall (Fig. 1), and 33-gauge needles are too flexible to yield reliable injections (R. E. Sievers, unpublished data). Furthermore, the mouse heart rate is ~500–600 beats/min. It is thus difficult to avoid entering the ventricular cavity, and no method to date has been available to confirm that the injection is intramyocardial rather than intraventricular. It has been proposed that this technical challenge is responsible for at least some of the disparate results of experiments based on direct injection of bone marrow cells into the mouse myocardial wall (9, 17) and is recognized as a technical hurdle in experiments involving direct injection of viral vectors (27). The second problem with the injection of cells within hours after infarction is its potentially limited clinical applicability. Access to bone marrow cells may not be immediately available in a patient experiencing an acute myocardial infarction. The acute administration of multiple antithrombotic agents will increase the risk for early bone marrow aspiration. Furthermore, some types of potentially therapeutic cells require time in culture. As such, patients would need to receive cell therapy days or weeks after the ischemic event. However, performing surgery on a mouse to induce the infarct and then repeating the surgery to expose the heart several days later are technically very difficult due to scar tissue formation and result in high mortality rates. Thus an approach is needed whereby cells can be injected into the myocardium in an accurate targeted fashion, without surgery, and in a clinically relevant time frame such as days or even weeks after a myocardial infarction.

High-resolution ultrasound systems have been used to study cardiac development and blood flow during mouse embry-
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Fig. 1. Comparison of standard needle bevel length to myocardial wall thickness. A representative frozen section of a mouse heart stained with hematoxylin and eosin and overlaid with the tips of 27- and 30-gauge needles is shown. Scale bar = 1 mm.

scanhead probe were aligned before the injection procedure such that the needle was at ~30° to the mouse table surface. After alignment was confirmed, the needle was retracted from the ultrasound field of view with the use of the micromanipulator, and the mouse was moved into position for echocardiographic visualization as described in Echocardiography. Special care was taken to visualize the targeted region of the myocardial wall, ensuring that its position matched the previously aligned needle position. The needle was advanced with the use of the micromanipulator under echo guidance through the body wall in a substernal diaphragmatic approach until the needle tip was in the desired location within the heart. The Optison-microsphere mixture (5–10 μl) was injected, and the accumulation of Optison was followed by ultrasound and documented as video clips on the Vevo 660 computer.

Echo-guided and conventional injection of dye into the myocardium. Evans blue dye (3%; J. T. Baker, Phillipsburg, NJ) was mixed 1:1 with Optison, and 5 μl of the mixture were then injected into the myocardium either under ultrasound guidance as described in Echo-guided injections of tracers into the myocardium or under direct visualization after surgery as described for induction of myocardial infarction. For open-chest injections, the hearts were pulled partially out of the chest cavity during injection and subsequently returned to their normal position. All mice were maintained under anesthesia for ~15 min after injection. The hearts were then harvested and cut in half, and the tissue was subjected to two successive incubations in formamide (1 day each, 1.25 ml total) at 55°C with gentle agitation to completely extract the dye (28). The two formamide volumes were combined, and the aliquots were then read spectrophotometrically in 96-well format in triplicate at 610 nm. Total theoretical yields were determined by incubation of un.injected hearts in formamide in parallel with the dye-injected hearts and by the addition of 5 μl of the same dye-Optison mixture after the hearts were processed. Standard curves were generated by reading a dilution series of the resulting mixture at 610 nm. Absorbance readings from all experimental groups were normalized to the standard curve, and resulting numbers were expressed as percentages of total dye injected ± SD. Statistical significance was determined by ANOVA followed by Dunnett’s T3 post hoc analysis with the use of SPSS software (SPSS, Chicago, IL); a p value <0.05 was considered significant.

Induction of myocardial infarction. Mice were anesthetized by inhalation of 2% isoflurane. The trachea was intubated and connected to a small animal volume-controlled ventilator (model 683, Harvard rodent ventilator; Harvard Apparatus, South Natick, MA). The heart was then exposed via a sternotomy with the use of a small retractor. A 7-0 suture was placed in the anterior myocardium at ~30% of the length of the heart proximal to the apex such that the left coronary artery was completely ligated. The incisions were then closed with interrupted sutures, and the endotracheal tube was gently retracted after voluntary respiration was restored.

Cell implantation. Mouse primary myoblasts expressing the β-galactosidase gene (lacZ; Ref. 19) were cultured as previously described (4) and suspended in PBS containing 0.5% BSA. Injections consisting of 5 x 10⁵ cells in 5 μl were performed with ultrasound guidance as described in Echo-guided injections of tracers into the myocardium.

Tissue processing and analysis. Animals were deeply anesthetized by inhalation of 5% isoflurane, and hearts were excised and frozen in a bath of isopentane and dry ice. Hearts were stored at ~80°C and sectioned with the use of a cryostat at 14 μm thickness. For observation of fluorescent microspheres, the sections were viewed dry without coverslip on a Nikon Eclipse E800 microscope with the use of a Hoechst filter set and a x10 objective. Images were captured with a Retiga charge-coupled device camera with the use of Openlab software (Improvision, Lexington, MA) and were pseudocolored green for maximum visibility. Faint brightfield images of the same sections were also obtained, and the two images were then merged. Subsequently, these slides were stained with hematoxylin and eosin and photographed again for comparison with the fluorescence image. For

MATERIALS AND METHODS

Echocardiography. All animal procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California at San Francisco. Male C57BL/6 mice (6–8 wk old) were anesthetized by inhalation of 1.5% isoflurane. Masking tape was used to anchor them to a positionable platform in a supine position. Long-axis echocardiography was accomplished with a Vevo 660 system (VisualSonics, Toronto, Canada) in B mode with the use of a 600 series real-time microvisualization (RMV) scanhead probe. The chests of the mice were shaved and prepped with povidone-iodine solution. The echocardiography scanhead probe were aligned before the injection procedure such that the needle was at ~30° to the mouse table surface. After alignment was confirmed, the needle was retracted from the ultrasound field of view with the use of the micromanipulator, and the mouse was moved into position for echocardiographic visualization as described in Echocardiography. Special care was taken to visualize the targeted region of the myocardial wall, ensuring that its position matched the previously aligned needle position. The needle was advanced with the use of the micromanipulator under echo guidance through the body wall in a substernal diaphragmatic approach until the needle tip was in the desired location within the heart. The Optison-microsphere mixture (5–10 μl) was injected, and the accumulation of Optison was followed by ultrasound and documented as video clips on the Vevo 660 computer.

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higher-magnification images of small deposits of fluorescent microspheres in the right ventricular (RV) cavity, the fluorescent image of the microspheres was superimposed on a darkfield image of the tissue pseudocolored red for purposes of contrast. For visualization of lacZ gene expression, sections were cut at 10\mu m, stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) solution as described previously (23), and then stained with hematoxylin and eosin.

RESULTS

Echo-guided percutaneous injections into myocardium. Our first aim was to determine whether a needle tip could be visualized in the myocardial wall by ultrasound with sufficient resolution to guide injections to specific regions. After positioning and alignment of the needle, ultrasound probe, and mouse as described in MATERIALS AND METHODS, we attempted to use the system to document the progression of the needle as it traversed the body cavity and entered the myocardium. As shown in Fig. 2 (more clearly demonstrated in the online data supplement video 1, available at http://ajpheart.physiology.org/cgi/content/full/00164.2005/DC1), we were able to recognize all relevant structures and to guide the needle into the myocardial wall without puncturing the left ventricular (LV) cavity.

To correlate the echocardiographic image to the actual needle position, we developed a dual injectable tracer consisting of a 1:1 mixture of echo contrast agent (Optison) and 0.2-\mu m fluorescent microsphere suspension. Fourteen mice were injected in this fashion in several groups as described in Calibration of targeting specific regions of the myocardium. During guided injection into the LV free wall, the Optison was visible by ultrasound as a concentration of echo-dense material that accumulated at the injection site. After the hearts were sectioned, the actual injection site was easily located with the use of a fluorescence microscope to visualize the microspheres. Figure 3 shows two representative injections in which we were able to observe the Optison accumulating in the LV free wall and then confirm our interpretation by histology. A control injection was also performed in which Optison without fluorescent microspheres was injected into the myocardium. After the heart tissue was sectioned, no fluorescence was detected.

Fig. 2. Visualization of needle with use of high-resolution echocardiography. Three frames from an ultrasound video clip are shown here, documenting the echo-guided insertion of a 30-gauge needle (small arrows) through the closed chest of a mouse into the myocardial wall near the apex of the left ventricle (LV; pink bracketed lines denote wall thickness). Needle tip (large arrow) is shown before penetration of the heart (a), halfway inserted (b), and fully inserted (c) into the myocardial wall. Mouse is on its back with its head facing to the right. Anatomical details and needle show up as white against a dark background. Scale bar = 5 mm. Cartoon illustrates spatial relationship of needle to myocardial wall. Ultrasound image is easier to interpret in the full motion video clip, which is shown in video 1 in the online data supplement (available at http://ajpheart.physiology.org/cgi/content/full/00164.2005/DC1).

Fig. 3. Tracking of echo-guided injections into myocardium with use of a mixture of ultrasound contrast and fluorescent microspheres. (a–d, e–h): subsequent injections in two different hearts that successfully targeted the LV free wall. In each row, the first echocardiogram is preinjection and the second is postinjection; third panel is a section of that heart stained with hematoxylin and eosin, and the fourth panel is the fluorescence (green) of the microspheres superimposed with a faint image of the section, which is the same as that shown by hematoxylin and eosin. Ultrasound images are easier to interpret in the full motion video clip, especially in the case of e and f; these are shown in videos 2 and 3 in the online data supplement (available at http://ajpheart.physiology.org/cgi/content/full/00164.2005/DC1). RV, right ventricle. Small arrows, needle; large arrows, position of injection before and after Optison accumulation; red lines, LV free wall. Note that the echocardiograms and the histology are oriented perpendicular to each other. Scale bar in c = 1 mm.
anywhere in the heart (data not shown), confirming that any fluorescence observed after the dual tracer injections was not due to the Optison or tissue damage-induced autofluorescence but was instead due to the injected microspheres.

 Calibration of targeting specific regions of the myocardium. The approach of using the Optison-microsphere mixture as an injectable tracer was instrumental in the initial calibration of our targeting method. This strategy was an essential part of our development of this approach and is a logical first step for other investigators who set up similar systems. With practice, we improved calibration of the ultrasound image to actual needle position with the following learning curve: our initial attempt to guide injections to the LV free wall of noninfarcted hearts (four animals) resulted in two septum injections and two RV injections; our second attempt (three animals) resulted in two septum/apex injections and one LV free wall injection; our third attempt (two animals) resulted in two successful LV free wall injections; and our fourth attempt (five animals) resulted in five successful LV free wall injections. Significantly, no animals died as a result of any of the echo-guided closed-chest injections.

In our initial attempt to target injections to the LV free wall, it was unclear how to interpret the ultrasound image, and we injected into unanticipated regions of the myocardium. However, the ability to visualize the injection site by fluorescence microscopy made it possible to reanalyze the echocardiographic data and confidently guide injections to desired locations in the subsequent attempts; this process is documented in Fig. 4. Figure 4, a-d, shows an injection in the interventricular septum rather than the LV free wall. In another early unsuccessful attempt, tracer was injected into the RV cavity because the mouse was positioned such that the RV cavity was partially overlaying the LV free wall. The ultrasound images demonstrated a transient crescent-shaped, echo-dense Optison cloud that cleared immediately (Fig. 4, e–i). In this case, fluorescent microspheres were not present in the myocardium; however, some were detected in a thrombus within the RV cavity. Further attempts included repositioning of the mouse with a slight rightward rotation, which proved essential to LV free wall access. These early attempts demonstrated that the sudden appearance of Optison followed by its immediate disappearance is a hallmark of a cavity injection, in contrast to the slower accumulation and retention of the Optison when it is successfully injected into the myocardium. (Compare online data supplement videos 2 and 3 with video 4 to witness the difference in kinetics.)

A major advantage of echo guidance is the avoidance of inadvertent injection into the LV cavity. To examine the distribution of tracer after such an intracavitary injection, we intentionally guided the needle into the LV cavity and injected the dual tracer (Fig. 5). As expected, there were no fluorescent microspheres detected in the myocardium at low magnification. However, despite the intentional loss of the injected material, higher magnification examination revealed that very small pockets of fluorescent microspheres had lodged in the myocardium, possibly along the needle track (Fig. 5, e and f). If unguided injections of cells are accidentally lost in the LV cavity, the detection of any cells in the myocardium could potentially be misinterpreted as indicative of a successful injection. The investigator in this case may be unaware that most of the injectate was lost in the blood pool.

Comparison of guided closed-chest injections versus open-chest injections. To determine whether guided closed-chest injections could deliver myocardial levels of injectate comparable to the open-chest technique, we injected a mixture of dye and Optison with the use of both approaches, recovered the dye, and measured the amount that had been injected into the tissue. Six mice were injected intramyocardially under surgical visualization and were compared with seven mice that received guided closed-chest injections. As negative controls, two mice received guided injections into the LV cavity (see Calibration
of targeting specific regions of the myocardium), and two more mice received guided injections of Optison alone into the myocardium (no dye control). Three positive controls were also performed in which dye was added to the samples immediately before quantitation rather than being injected into the tissue (see MATERIALS AND METHODS). With the use of the negative and positive controls as reference points, along with standard curves generated from the positive controls, we were able to quantitate the percentage of injected dye that was successfully delivered to the myocardium and retained there.

As shown in Fig. 6, both the open- and closed-chest approaches resulted in large blue regions in the myocardial wall. Interestingly, dye was observed in large drainage vessels within minutes of both kinds of injections, presenting an additional mechanism for loss of injectate regardless of successful injection. On the basis of this observation, we were careful to allow a consistent length of time between injection and tissue harvest to prevent differences in dye loss into the vasculature between groups. Furthermore, because the hearts of the open-chest group were partially pulled out of the chest cavity during injection, we returned them to their normal position to avoid any potential effects of temperature-related vasoconstriction and vessel deformation on the rate of clearance.

The results are shown in Fig. 7. The first two of the seven closed-chest injections did not show any dye accumulation. Notably, the use of ultrasound visualization made us aware of the problems with these injections even as they were being performed, underscoring the advantage of this approach. In an experiment involving therapeutic injections, we would know to remove these mice from the experiment at the beginning. Therefore, although we have still included these two mice in our results, we consider them outliers. The remaining five closed-chest injections, which we already believed had worked on the basis of the ultrasound imaging, resulted in less variability than the open-chest group and comparable dye retention (8.2 ± 1.9% vs. 8.0 ± 3.5%; P = 1.0). The closed-chest group had significantly higher values than both negative control groups (P = 0.008 for each), as did the open-chest group (P = 0.04 for each). Significantly, no injection with the use of any approach resulted in recovery of >13% of the dye that was injected, demonstrating that partial clearance in the vasculature or potential backflow through the needle track after needle withdrawal is a potentially confounding factor in any therapeutic myocardial injection strategy. However, it was clear that the guided closed-chest injections had not punctured the LV cavity, and to the extent that material can be injected into beating cardiac muscle, this approach rivaled the open-chest approach in the amount of injectate delivered, with less variability.

Targeting of closed-chest injections to myocardial infarcts. The ultimate aim of the echo-guided injection approach is to guide injections of therapeutic agents such as stem cells to the infarct or its border zone. To demonstrate that cell implantations in this manner are feasible, we created infarcts in six mice and used high-resolution ultrasound to guide closed-chest injections of Optison and fluorescent microspheres to the perinfarct border zone 3 days postinfarct. As was the case with targeting the noninfarcted LV free wall described in Calibration of targeting specific regions of the myocardium, one preliminary experiment (n = 6 hearts) was necessary before we could reliably interpret the ultrasound visualization of the needle insertion into a second set of infarcted hearts (n = 6).

Accordingly, our first attempt resulted in three out of six successfully targeted injections, and our second attempt resulted in a

### Fig. 5
Intentional injection into the LV cavity resulting in potentially misleading accumulation of material in the myocardium. a–c: three frames from an ultrasound video clip document the injection of Optison and fluorescent microspheres into the LV cavity. Optison is transiently present in b and disappears <1 s later in c. d: hematoxylin and eosin-stained section of that heart. e and f: two sections ~300 μm apart at high magnification of the boxed area in d. A small region of fluorescent microsphere accumulation is observed (arrows) despite intentional loss of the injected material in the cavity. Scale bar = 1 mm in d and 500 μm in e and f.

### Fig. 6
Comparison of open-chest and closed-chest injections of dye. Representative hearts are shown after direct open-chest injection and echo-guided, closed-chest injection of Evans blue, visible as a large patch of dark blue dye. Arrows point to drainage vessels filled with dye (enlarged with enhanced contrast in inset). Main panels are at equal magnification (larger size of the heart on left is probably due to its having been photographed before it had stopped beating).

### Fig. 7
Quantitation of injectate in the myocardium after open-chest versus closed-chest injections. Values are normalized to percentage of injected dye that is recovered after injection and tissue processing. Circles represent individual mice. Bars show the mean values of both groups. Open circles show outlying unsuccessful closed-chest injections; dotted line shows the mean value for closed-chest animals, including the unsuccessful outliers (see text).
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Fig. 8. Targeting of injections to the infarct border zone. a and b: successful targeting of a closed-chest injection of Optison and fluorescent microspheres to the basal region of an apical infarct, 3 days postinfarction. Wall thinning is apparent in this heart. c–f: two different sections of another 3-day infarcted heart ~1.5 mm apart. Wall thinning is not apparent in this heart; however, the infarct can be recognized by the disruption of normal hematoxylin and eosin staining (arrows). Injection position is peri-infarct in both sections. g–i: successive sections ~300 μm apart from a heart injected with β-galactosidase (lacZ)-expressing myoblasts 7 days postinfarction and harvested 10 days later. Sections progress from apex to base; infarct scar is at the top. Blue color is from 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining to detect lacZ gene expression. Scale bar = 1 mm.

sulted in six out of six successes (two representative hearts are shown in Fig. 8, a–f).

For further confirmation that targeted injection of cells is feasible, we created infarcts in two immunodeficient SCID mice, and at 7 days postinfarct, we targeted injections of mouse skeletal muscle myoblasts expressing the lacZ reporter gene (19) to the infarct border zone. Two injections were performed in each heart. These myoblasts are known to survive and express lacZ after implantation into noninfarcted mouse myocardium (10) and serve as better characterized surrogates for the different populations of bone marrow-derived putative stem cells that will ultimately be examined with this system. We harvested the hearts 10 days postimplantation to allow inflammation to subside and the cells to engraft. Tissue sections stained with X-gal identified implanted cells in the infarct border zone in each case (one case is shown in Fig. 8, g–i). These results demonstrate that high-resolution echocardiographic guidance is a feasible approach to accurately target clinically relevant intracardiac injections in the mouse.

DISCUSSION

This report is the first description of echo-guided percutaneous intramyocardial injections, which can replace open-chest injections. Major advantages of this approach are that injections can be performed at clinically relevant times, that multiple injections are possible, and that the injectate is delivered at amounts comparable to open-chest injections. Despite the relatively noninvasive approach, we have demonstrated that the placement of the needle into the myocardial wall is accurate and predictable, that guided closed-chest injections and direct open-chest injections can deliver comparable amounts of injectate, and that injections into infarcted hearts can be targeted to the peri-infarct border zone many days after the infarct. These conditions are made possible by the ability to visually follow the progression of the needle as it traverses the body cavity and enters the myocardial wall, preventing it from puncturing the LV cavity.

Despite the larger size of rats, the mouse continues to become increasingly important as a small animal model of myocardial infarction due to its highly developed genetics and the existence of useful transgenic strains. However, its small size has imposed severe limitations on the utility of the mouse as a viable preclinical model for the therapeutic injection of plasmid DNA, recombinant viruses, myoblasts, and putative stem cells. Through the use of high-resolution echocardiography as described in this study, these limitations are substantially diminished, and myocardial injection therapies can now be tested with the use of naturally occurring mutant and transgenic strains that have altered cardiac function, visibly marked cells, or conditional deletions (1, 15, 16). Moreover, injections can be performed days or weeks after surgical induction of an infarct, allowing experiments to be designed involving injections at clinically relevant times and even on multiple days. Without ultrasound guidance, these experiments would only be possible through a second surgical procedure, leading to an extremely high mortality rate. However, none of the mice in these experiments died as a result of the guided percutaneous injections. In addition, this high-resolution echocardiographic system permits analysis of LV wall thickness, LV internal dimensions, and ejection fraction with greater accuracy than systems currently in use for mouse echocardiography (14).

However, it is not sufficient to target the myocardium; it is necessary to recognize the specific region of the myocardium into which the needle has been inserted. To this end, our combined ultrasound/fluorescent tracer was extremely useful in determining how to locate specific sites for injection. We recommend that other investigators who attempt to use this system try a similar approach to orient themselves. Optison is an ultrasound-opaque contrast agent that is used in clinical echocardiography for improved visualization of myocardial function. The 0.2-μm fluorescent microspheres are large enough that they remain trapped in tissue, but small enough that they form an even suspension, and have been used for vascular tracing of potentially leaky blood vessels (21, 22). They are also bright enough to be visible with a 1× objective, which transmits too little light to be used for typical immunofluorescence microscopy. Together, the Optison and fluorescent microspheres comprise a dual tracer that can be followed through the injection process and detected afterward by microscopy. Because no biological engraftment is necessary, as would be the case with the injection of marked cells, the results of a series of test injections can be assessed immediately after the injection, simply by harvesting and sectioning hearts, and with no requirement for fixing or staining steps unless a counterstain is desired afterward. In this way, we were able to calibrate the echocardiographic image during injection with the...
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actual injection site, allowing us to perfect our needle-positioning. We view this as a rapid and straightforward but essential first step in the deployment of such a system.

A similar approach has been described recently that uses magnetic resonance (MR) imaging to guide injections into rat myocardium (5). However, the use of MR imposes several limitations that restrict its efficacy in the smaller mouse model. The spatial resolution of MR with the use of a 0.5-T magnet, as was used in that study, would be >0.5 mm, whereas the resolution of the VisualSonics ultrasound system is ~30 μm. Similar resolution can theoretically be obtained with MR with the use of a 7-T magnet; however, this would necessitate a specialized facility, in contrast to the ultrasound system, which can be mounted on an ordinary laboratory research bench. A 22-gauge needle was visible in the MR study as a rough shadow against a light background, whereas a 30-gauge needle shows up as a sharp bright line with an easily discernable tip during high-resolution ultrasound imaging. With respect to temporal resolution, the MR image was updated every 5 s, whereas ultrasound imaging with the probe used for guidance of injections has a frame rate of 30 frames/s, allowing truly real-time guidance of the needle into the myocardium. Electrocardiogram gating during MR also presents a difficulty for real-time guidance of the needle into the myocardium. Even without contrast agent, high-resolution echocardiography allows the position of the needle tip to be detected within the myocardial wall during injection of cells or other materials. Without echo guidance, injections intended for the myocardial wall may very likely pierce the endocardium and result in a significant loss of material into the LV cavity. Our results suggest that during such nonguided injections, even the loss of the majority of cells into the chamber can still leave enough cells in the myocardium that such injections may erroneously appear to have been successful. These findings may explain some of the inconsistencies encountered in the literature related to direct injection of bone marrow-derived cells into myocardium (3, 13, 17). Whereas some researchers have considerable experience with direct injections into surgically exposed mouse myocardium (20), this echo-guided percutaneous approach ensures that intramyocardial injections of cells or other potentially therapeutic material occur in the specific desired region of the myocardial wall while confirming that the material remains in the myocardium, and it allows the injections to take place at any clinically relevant time after the induction of myocardial infarction without a second surgery.

REFERENCES


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