Cell tracking using iron-oxide fails to distinguish dead from living transplanted cells in the infarcted heart

Magnetic Resonance in Medicine. 2009; in press

Winter EM¹, Hogers B¹, van der Graaf LM¹, Gittenberger-de Groot AC¹, Poelmann RE¹, van der Weerd L¹,²

¹ Dept. of Anatomy & Embryology, ² Dept. of Radiology, Leiden University Medical Center, Leiden, The Netherlands
Abstract

Background and methods
Recently, debate has arisen about the usefulness of cell tracking using iron-oxide-labeled cells. Two important issues in determining the usefulness of cell tracking with MRI are generally overlooked, firstly the effect of graft rejection in immunocompetent models, and secondly the necessity for careful histological confirmation of the fate of the labeled cells in the presence of iron-oxide. Therefore, both iron-oxide-labeled living as well as dead epicardium-derived cells (EPDCs) were investigated in ischemic myocardium of immunodeficient NOD/scid mice with a 9.4T MRI until 6 weeks after surgery, at which time point immunohistochemical analysis was performed.

Results
In both groups, voids on MRI scans were observed that did not change in number, size or localization over time. Based on MRI, no distinction could be made between living and dead injected cells. Prussian blue staining confirmed that the hypointense spots on MRI corresponded with iron-loaded cells. However, in the dead-EPDC recipients, all iron-positive cells appeared to be macrophages, while the living-EPDC recipients also contained engrafted iron-loaded EPDCs.

Conclusions
Iron-labeling is inadequate for fate determination of transplanted cells in the immunodeficient host, since dead cells produce an MRI signal indistinguishable from incorporated living cells.
Introduction

In the last decade a large amount of cardiovascular research has focused on cellular regeneration therapy of the infarcted heart. A wide variety of cell types has been tested in pre-clinical experimental animal models as well as in clinical trials, with different results (see \(^2\) for review). With the broad application of experimental stem cell transplantation, \textit{in vivo} tracking of transplanted cells becomes more and more mandatory. Magnetic resonance imaging (MRI) provides the possibility for non-invasive longitudinal imaging of cellular distribution and migration when cells of interest are loaded with iron before transplantation into the infarcted heart. Superparamagnetic iron-oxide (SPIO) particles are good candidates for this purpose: labeling is relatively easy and does not affect proliferation and differentiation capacity \textit{in vitro} \(^3,4\). It was recently demonstrated that labeling of mesenchymal stem cells (MSCs) with SPIO particles (Ferumoxide or Endorem) does not affect therapeutic efficacy of these cells: iron-labeled MSCs kept their potential to attenuate left ventricular dilatation and dysfunction after myocardial infarction (MI) \(^5\).

Several studies demonstrated that iron-labeled cellular transplants were visible with MRI and could be followed over time \(^3,4,6,7\), even when a magnet of clinical field strength was used \(^4,8\), although detection limit was low and only high numbers of iron-loaded cells were visible \(^9\). It was only assumed that these hypointense spots on the MRI images actually represented the transplanted living iron-loaded cells. It was not verified whether the transplanted cells were indeed present in histological sections, and whether MRI signal was really generated by the original transplant and not by e.g. macrophages that had phagocytosed the iron-containing cells. The first study \(^5\) that addressed this issue reported that the MRI signal originally generated by iron in transplanted MSCs appeared to be present regardless of the existence of these cells, which was later confirmed by Terrovitis \textit{et al} \(^10\) who demonstrated, like Amsalem \textit{et al} had done before, that iron-loaded macrophages created the signal rather than the iron-loaded living transplanted cells.

As suggested by Sadek and Garry in a comment to the study of Amsalem \textit{et al}, the results should be extended by studies in immunocompromised animals \(^11\). In the previous studies, cardiac-derived stem cells (CDCs) or MSCs, which are not immunoprivileged, were harvested from ‘syngeneic’ rats and transplanted into other inbred immunocompetent rats \(^5,10\). However, rats can not, unlike mouse donors from same inbred strain, be considered really syngeneic but rather allogeneic \(^5\), implying that the cells could simply be rejected by the host, and thus that the concern regarding the utility of SPIOs in cardiac cell therapy applies only to comparable studies with non-autologous cells and immunocompetent animals. Sadek and Garry, therefore, pleaded for similar experiments with either autologous cell transplantation in normal animals, or with allogeneic stem cell transplantation into immunodeficient animals \(^11\). It could very well be that iron-labeled transplanted cells are not rejected in those settings or that the clearance pattern is altered in such a way that the hypointense signals from iron-oxide largely correspond to stem cell engraftment.

We transplanted both dead and living iron-labeled human adult epicardium-derived cells (EPDCs) into the infarcted heart of non-obese diabetic severe combined immunodeficient (NOD/scid) mice and tracked the resulting signal voids over six weeks. EPDCs are demonstrated to engraft in the infarcted cardiac area of NOD/scid mice, and to improve cardiac function and protect the ischemic tissue after transplantation into the infarcted myocardium \(^12\) making them both suitable and interesting for \textit{in vivo} tracking experiments. By transplanting both dead and living iron-labeled cells, we sought to discriminate these two groups based on signal intensity, homing pattern, or clearance of iron-oxide by macrophages over time. We aimed to distinguish live from dead cells by following the hypointensities over time, seeing either homing of living cells to the infarcted area, or clearance of dead cells and iron-oxide by macrophages.
Methods

EPDCs were isolated from human adult atrial appendages as previously described\textsuperscript{12,13}. For iron-oxide-labeling EPDCs were seeded in a density of $5 \times 10^4$ cells/cm$^2$ and incubated overnight with a mixture of $0.03 \text{ mg iron/cm}^2$ (Endorem, Guerbet, Gorinchem, the Netherlands) and $0.075 \mu l/cm^2$ Metafectene (Biontex, Munchen, Germany) in serum free culture medium (labeling efficiency > 95%). To kill iron-loaded EPDCs, while taking care not to disturb cell membrane integrity, trypsinized cells were incubated for 24 h under nitrogen (>98% dead cells). MI was created by ligation of the left anterior descending coronary artery (LAD) which resulted in significant increase of left ventricular chamber volumes\textsuperscript{12,14}. Immediately after occlusion of the LAD, a total number of $4 \times 10^5$ iron-labeled dead or living EPDCs suspended in 20 μl culture medium was injected into the ischemic myocardium of immunodeficient NOD/scid mice as described before (n=2 per group)\textsuperscript{12}. MRI was performed subsequently at day 5, 14, 28, and 42 after MI with a vertical 9.4T magnet (Bruker, Ettlingen, Germany) supplied with an actively shielded Micro2.5 gradient of 1T/m and a 30m transmit/receive birdcage RF coil, using an electocardiogram (ECG) and respiratory triggered Flash sequence. As described previously\textsuperscript{12,14}, animals were anesthetized with isoflurane (4% for induction and 1.5-2% for maintenance) in a mixture of oxygen and medical air (1:1) with a flow of 0.6 L/min. Neonatal ECG electrodes (3M, Red Dot\textsuperscript{TM}), attached to the left fore- and right backlimb, and a respiration detection cushion placed under the thorax, were used for monitoring of and triggering on ECG and respiration signals. The depth of anesthesia was continuously regulated to maintain stable respiration rate during the experiment. Body temperature was maintained at 35 °C. After three-dimensional plane localization, four chamber views (coronal), short-axis views (transversal), and views parallel to the interventricular septum (sagittal) were acquired (see Figure 1). In each direction, 16 contiguous slices of 0.5 mm thickness were made, together covering the entire heart. Further parameters were a repetition time (TR) of 97 ms, an echo time (TE) of 2 ms, and a flip angle of 20°. Field of view (FOV) was 25.6 x 25.6 mm, the matrix 256 x 256, and the number of averages (NEX) was 12. All images of the three different planes (see Figure 1) were assessed for iron generated hypointense spots within the cardiac wall by two independent observers blinded to treatment. Spots were evaluated for their number, size, localization and distribution in the heart, using the valves, papillary muscles and the structures surrounding the heart as reference points.

![Figure 1](image_url)

Figure 1. Representative images (at six weeks after surgery) showing the three different planes, coronal (a), transversal (b) and sagittal (c), used for determination of the iron generated voids present in the heart. H: head, F: feet, L: left, R: right, A: anterior, P: posterior.
For histology hearts were harvested at day 43, one day after the last MRI scans were performed. Prussian blue staining was performed on formalin-fixed paraffin-embedded hearts to compare physical iron localization with hypointensities on the MRI scans. To identify the cells that had taken up the iron particles, we performed antibody staining for the macrophage marker CD68 (MCA1957, Serotec, Raleigh, NC, US) in sections consecutive to those stained with Prussian blue. Primary antibody was incubated overnight. As secondary antibody biotinylated rabbit anti rat (BA-4001, Vector Laboratories, Burlingame, CA, USA) was used. Staining was visualized using Vectastain™ ABC Alk-Phosphatase kit (AK-5200, Vector Laboratories) and Vector Red Substrate (SK5100, Vector Laboratories). Notably, visualization of antibody signal using the horseradish peroxidase reaction with 3,3’-diamino-benzidine tetrahydrochloride (DAB) can not be used since the ‘redox-active’ iron in the Endorem containing cells reacts with the DAB, resulting automatically in DAB precipitation in all iron containing cells 15. Sections were briefly counterstained with Mayer’s hematoxilin.

Results and Discussion
Large hypointensities, generated by the iron particles, were clearly visible in all MRI scans of each heart observed, scattered throughout the ischemic left ventricular wall. It was remarkable that no differences could be observed between dead and living cell recipients regarding size, number, localization or distribution of the voids (Figure 2). Moreover, variation over time within each animal was absent as well: the prominent voids remained unchanged from day 5 until day 42 (Figure 2), as has been reported before by some previous studies although not given much attention 7,16. We confirmed with a Prussian blue staining that the voids on the MRI images corresponded with iron particles present in the heart tissue (Figure 3). Iron was only present in intact cells with a nucleus and not free in the extracellular matrix.

It should be noted that the commonly employed reaction of horseradish peroxidase with DAB may not be used for antibody stainings in iron containing sections since iron itself reacts with the DAB substrate resulting in a false positive DAB precipitate 15. Although some studies acknowledged this problem and used other labeling techniques 9,10, others did not notice it 5,16-18 and their results regarding the properties of the iron-positive cells need thus to be taken with caution. Regarding the findings of other studies that macrophages had phagocytozed the iron-loaded cells, only the study of Terrovitis et al 10 convincingly demonstrated with fluorescent double labeling that iron colocalized with macrophages in the heart. In that study, however, immunocompetent rats were used for the transplantation experiments, leaving the question whether results are also true for immunocompetent recipients, used by many stem cell studies.

We investigated the identity of the iron-containing cells in the immunocompromised host, using a precipitation reaction with alkaline phosphatase and red substrate which we verified not to react with iron like DAB does. In the dead-EPDC recipients, all iron-containing cells were positive for the macrophage marker CD68 (Figure 3a, d) demonstrating that the iron particles were phagocytozed by macrophages. The group that had received living EPDCs contained mainly CD68 positive iron-loaded macrophages (Figure 3b, e) but also some CD68 negative iron-loaded engrafted EPDCs (Figure 3c, f) which were, with their elongated shape, different in morphology from the round and large macrophages. This corresponds well to earlier studies where non-labeled EPDCs were found to engraft in the ischemic myocardium 12.

We demonstrated for immunocompromised NOD/scid mice, lacking adaptive immunity and having only altered innate immunity 19,20, that transplantation of iron-loaded allogeneic cells into the infarcted hearts results in an MRI hypointensity for at least six weeks, which does not change in size or localization over time. There was no discrimination between initially engrafted living cells...
Figure 2. Transplantation of living (a-h) and dead (i-p) iron-labeled EPDCs results in large hypointensities (voids, arrows) in the ischemic left ventricular wall. Number, size and localization of the voids are comparable between groups and do not change over time (day 5 until week 6). Sagittal views: a-d for living EPDCs and i-l for dead EPDCs; Short-axis views: e-h for living EPDCs and m-p for dead EPDCs.
and dead cells, the latter would be unable to integrate in the cardiac tissue. The signal was thus not dependent on engraftment of the transplanted cells, although free iron particles injected into the heart are cleared rapidly and will escape observation 3.

In conclusion we can state that iron-labeling is not recommended for tracking of living cells over time in any of the above described transplantation models – neither for immunocompetent nor for the immunodeficient host, as there is no discrimination between healthy, initially successfully engrafted, stem cells and dead stem cells phagocytosed by macrophages within the heart. The iron, inserted into the heart within the transplanted cells, remains present at the site of injection notwithstanding incorporation into macrophages, and was not cleared from the injection site at six weeks after transplantation as we hypothesized. Iron-labeling can, however, be very informative to evaluate whether stem cells were successfully injected into the organ of interest, to visualize the area of injection, and to pursue this site of injection over time.

Figure 3. Consecutive sections show that iron containing cells (blue) in dead-EPDC recipients (a) are positive for the macrophage marker CD68 (pink) (d), demonstrating that the macrophages had phagocytosed the iron. For living-EPDC recipients most iron containing cells (b) were positive for CD68 (e). These cells were large and round, like observed for the dead-EPDC recipients. However, in the living-EPDC recipients some differently shaped elongated iron containing cells (c) were observed which were negative for CD68 (f). Scale bars: 20 μm. Arrows indicate CD68 positive cells, arrowheads indicate Prussian blue positive but CD68 negative cells.