In Vivo and Ex Vivo MR Imaging of Slowly Cycling Melanoma Cells

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Slowly cycling cells are believed to play a critical role in tumor progression and metastatic dissemination. The goal of this study was to develop a method for in vivo detection of slowly cycling cells. To distinguish these cells from more rapidly proliferating cells that constitute the vast majority of cells in tumors, we used the well-known effect of label dilution due to division of cells with normal cycle and retention of contrast agent in slowly dividing cells. To detect slowly cycling cells, melanoma cells were labeled with iron oxide particles. After labeling, we observed dilution of contrast agent in parallel with cell proliferation in the vast majority of normally cycling cells. A small and distinct subpopulation of iron-retaining cells was detected by flow cytometry after 20 days of in vitro proliferation. These iron-retaining cells exhibited high expression of a biological marker of slowly cycling cells, JARID1B. After implantation of labeled cells as xenografts into immunocompromised mice, iron-retaining cells were detected in vivo and ex vivo by magnetic resonance imaging that was confirmed by Prussian Blue staining. Magnetic resonance imaging detects not only iron retaining melanoma cells but also iron positive macrophages. Proposed method opens up opportunities to image subpopulation of melanoma cells, which is critical for continuous tumor growth. Magn Reson Med 000:000–000, 2011. © 2011 Wiley-Liss, Inc.

Key words: cell tracking; cancer stem cells; melanoma; MRI; iron oxide particles

The low efficacy of some existing methods of treatment of human cancers has been attributed to the existence of cancer stem cells. The cancer stem cell model hypothesized the existence of a small subpopulation of cells with the capacity to recapitulate a tumor after conventional treatment. Cells with these properties were identified in breast (1), colon (2), or brain tumors (3). The following stem-like subpopulations of cells have been found in melanoma: CD20-positive cells (4), cells with high-efflux activity expressing high levels of the ABC multidrug resistant genotype (5–7), and label-retaining cells (4,5,8–10). These cell populations exhibit many overlapping properties but are not identical. The significance of label-retaining cells was recognized, and several methods have been established for their identification, the most common being labeling with PKH26/67 (11,12). These fluorescent lipophilic reagents are incorporated into cell membranes and after several cell divisions are diluted below the limit of detection. However, slowly cycling cells retain measurable levels of fluorescence over time (10). In our experiments, we are using the same concept of label retention but are targeting cells with magnetic resonance imaging (MRI) reagent, iron oxide particles, which has been shown capable of being detected at the single cell level (13).

The focus of this study is slowly cycling melanoma cells. Our laboratory identified a molecular marker, JARID1B, for slowly cycling cells (14). This protein is a member of the jumonji family of chromatin regulators (15,16). JARID1B is ubiquitously expressed in adult tissues, with peak expression levels in regenerative tissue such as testis and bone marrow (17). In spite of its presence in trace quantities in almost every cell, high expression of JARID1B occurs almost exclusively in cells with long doubling times. The chromatin modification capacity of JARID1B is mediated by histone 3K4 demethylase activity (18–21). The level and distribution of histone methylation have been associated with stem cell maintenance (22,23). In particular, demethylation of H3K4 by JARID1B plays a role in cell fate. In human embryonic stem cells, it blocks terminal differentiation (24) and mediates cell cycle arrest (25). In melanoma, only ~1% of cells exhibit high expression of JARID1B. Isolated JARID1B-positive cells produce progeny with highly proliferative capacity. Knocking down of JARID1B leads to an initial acceleration of tumor growth followed by exhaustion, which suggests that the JARID1B-positive subpopulation is essential for continuous tumor growth (14).

MRI provides an effective method for noninvasively tracking of implanted cells (26). The high efficiency of this technique is due to the high spatial resolution of MRI and the availability of efficient labeling techniques. To distinguish donor cells from host tissue, implanted cells are labeled with an MRI contrast agent in vitro and after transplantation can be identified in high resolution in vivo MR images. Research MRI scanners achieve a resolution of 100–200 μm for in vivo experiments, which can be further increased to 50–100 μm in ex vivo studies. The most widely used contrast agents for in vivo MRI detection of implanted cells are superparamagnetic iron oxide (SPIO) particles (13,26–30). These particles enter the cell by endocytosis and are generally sequestered in endosomal compartments. In the presence of a
magnetic field, the large microscopic magnetic moment of SPIO particles distorts the external magnetic field, reducing the $T_2/T_2^*$ relaxation times of the neighboring water molecules in the tissue and diminishing the local MR signal. The field distortions created by iron oxide particles encapsulated in a single cell extend much further than the diameter of the cell, which significantly facilitates the detection of the labeled cell (26). These reagents have been used for detection of single cells in vitro (31) as well as for in vivo (32). Iron oxide particles are available in various sizes. Micron-size iron oxide particles ([MPIO] particle diameters ~0.5–2.0 μm) were used for detection of individual cells (13). Ultra small superparamagnetic iron oxide particles ([USPIO] particle diameters ~5–50 nm) have been used for imaging the lymphatic system (33–35).

The objective of this study was to evaluate the feasibility of in vivo MRI detection of slowly cycling melanoma cells. We demonstrated that after initial uptake of the MRI contrast agent, normally cycling cells dilute iron as they divide, whereas slowly cycling cells preserve a higher intracellular concentration of the contrast agent. The presence of iron-retaining cells was detected in vivo and confirmed by ex vivo MRI and histological staining.

MATERIALS AND METHODS

Human Melanoma Cell Culture

Human melanoma cells (WM3734, WM3918, WM35, 1205Lu, and 963b) were plated at a concentration of 4 × 10^4/cm² in 2% FBS-substituted melanoma growth medium as reported earlier (4).

Labeling of Cells With Iron Oxide Particles

Three types of iron oxide particles were used in this study: USPIO (average diameter 30 nm) kindly donated by Dr. A. Tsourkas laboratory (Department of Bioengineering, University of Pennsylvania), SPIO (average size 150 nm) (Feridex, Berlex Imaging), and MPIO (average diameter 860 nm) (Bangs Laboratory). Iron oxide particles were mixed with cell medium at concentrations of 100 μg/mL for USPIO and SPIO particles and 45 μg/mL for MPIO particles. To facilitate cellular uptake, SPIO particles were preincubated with a freshly prepared solution of protamine sulfate (1 mg/mL) (Sigma) for 1 h (36). The growth medium was replaced with iron oxide containing medium, and melanoma cells were incubated overnight (27). Excess iron oxide particles were removed by vigorous washing with phosphate-buffered saline (PBS) 3–5 times and one time with heparin (10 U/mL) in PBS. After washing, cells were trypsinized, spun down, resuspended in PBS, and kept on ice.

Iron Measurement

The iron concentration per cell was determined using inductively coupled plasma mass spectroscopy on separate batches of labeled cells (University of Pennsylvania Toxicology Laboratory, New Bolton Center). Briefly, cells were resuspended in 1 mL of PBS and counted, digested with an equal volume of nitric acid overnight, and the resulting solution was submitted for standard inductively coupled plasma mass spectroscopy measurement. The concentration of iron per cell was calculated by dividing the total iron content in the sample by the total number of cells. These experiments were repeated thrice, and the average iron concentration was calculated.

Toxicity Test

To evaluate the effect of iron oxide particles on cell proliferation, four melanoma cell lines (WM3734, WM3918, WM35, and 1205Lu) were incubated with 100 μg/mL of USPIO, SPIO, and 45 μg/mL of MPIO particles overnight. After careful washing, 5 × 10^5 cells were seeded and allowed to proliferate. Because of optical absorption of iron particles at 566 nm wavelength, a traditional MTT test of cell viability was not utilized. Instead, after 5 days of proliferation, viable cells (Trypan Blue negative) were counted manually with a hemocytometer. The proliferation experiment was performed on each cell line at least five times.

The ability of iron-labeled melanoma cells to form three-dimensional colonies was also evaluated. Experiments were performed in standard 6-well plates. The bottom of the plate was coated with 1% agar and cell medium at a 1:1 ratio. The agar/medium mixture was allowed to settle in a vibration-free environment for 1 h. A single cell suspension of 5 × 10^3 cells was mixed with 0.7% agar and transferred onto the coated plate. Cells were allowed to proliferate for 2–4 weeks; 1.5 mL of fresh medium was added every 4 days. The number of colonies was counted under the microscope or from high-resolution pictures of the cell culture. Each experiment was performed thrice, and number of colonies grown from iron labeled and iron free cells was compared.

Flow Cytometry

Flow cytometry experiments were conducted to detect an iron-retaining sub-cell population and to demonstrate dilution of iron oxide particles due to cell division. Briefly, WM 3734 cells were labeled with USPIO and MPIO particles conjugated to a fluorescent dye. Flow cytometry for detection of iron-retaining cells was performed at 24 h, 11 days and 20 days after labeling. Dead cells were excluded by gating of the viable population.

Subcutaneous Cell Implantation

Animal procedures were conducted strictly according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care of the University of Philadelphia and the Wistar Institute. Three separate groups of animals were studied: mice injected with USPIO ($n = 8$), SPIO ($n = 6$), and MPIO ($n = 7$) labeled WM3734 cells. One million labeled cells (100 μL) were subcutaneously injected into the lower right flank of NOD/LtScidIL2Rγnull mice. The same number of control (unlabeled) cells was injected into the lower left flank of the same animal. Labeled and unlabeled cells were mixed with a Matrigel suspension at a ratio of 1:1.
Magnetic Resonance Imaging Experiments

Phantom Studies

Two-percent gelatin impregnate with 2 mM Gd-DTPA was used in phantom experiments. To demonstrate the feasibility of MRI detection of single MPIO particles, we diluted the original suspension (1.8 x 10^10 particles) multiple times and prepared a suspension of ~300 particles. Diluted particles were mixed with 2 mL gelatin and permitted to solidify. T2-weighted MR images of the agar block were acquired at 9.4 T with a 20 mm diameter NMR high-resolution liquid probe. Three-dimensional gradient echo images at different resolutions (58, 78, 117, 156, and 217 µm) were acquired with the following parameters: repetition time 0.5 s, echo time 5 ms, slab thickness = 2 cm, file of view 1.5 or 3 cm².

In Vivo Imaging

In vivo imaging of mouse tumors was performed using a 4.7 T horizontal bore magnet equipped with a 12 cm, 25 G/cm gradient insert [50 cm horizontal bore magnet interfaced to a Varian imaging console (Varian Inc.)]. Mice were anesthetized with 1% isoflurane in air during the MRI experiment. A home built linearly polarized 50 mm inner diameter birdcage coil was used for imaging. The core body temperature and ECG were monitored during the experiment using an MRI-compatible physiological monitoring unit (SA instruments Inc.). The body temperature of the animals was maintained at 37°C ± 1°C by blowing warm air regulated by a feedback loop through the magnet bore. In vivo two-dimensional (2D) gradient echo imaging was performed with the following parameters: repetition time = 400 ms, echo time = 4.5–10 ms, 4 scans, file of view of 3.9 x 1.8 cm², 256 x 128 matrix, producing an in-plane resolution of ~150 µm, and slice thickness of 0.5–1 mm.

At the end of the in vivo experiments, mice were sacrificed under deep anesthesia, fixed by transcardial perfusion with 30 mL of cold Dulbecco’s PBS followed by 30 mL of 4% paraformaldehyde. Tumors were removed and post-fixed for 5 days in 4% potassium ferrocyanide and 2% hydrochloric acid for 30 min in a 37°C water bath. The slides were then rinsed with distilled water and counterstained with nuclear fast red (Vector Laboratories) for 20 min.

Ex Vivo Imaging

Perfused tumor tissue was imaged in a 9.4 T vertical bore magnet interfaced to a Varian console. Mouse tumors (n = 15) were placed in an 18 mm ID sample holder and immersed in ~2 mL Fomblin (Ausimount) to avoid background signals. A 20-mm home built solenoid transmit/receive coil was used for imaging. Three-dimensional gradient echo imaging was performed with the following acquisition parameters: repetition time/echo time 100/10 ms, 28 scans, file of view ~2 x 1 x 1 cm³, 256 x 128 x 128 matrix, leading to an isotropic resolution of ~80 µm, and an acquisition time of ~2 h.

Image Analysis

The acquired 3D images were displayed using the “Image” (NIH image, http://rsb.info.nih.gov/nih-image/) program and reformatted into a stack of 2D slices.

Histology

At the end of the ex vivo MRI experiments, tumors were removed from the NMR probe, rinsed with PBS solution, cryoprotected by incubation in 5 M sucrose solution, embedded in OCT, and frozen on a bed of crushed dry ice. Five-µm-thick cryosections were prepared and stained with Prussian Blue. Another set of tumor tissues was embedded in paraffin.

Prussian Blue Staining for Detection of Iron

Slides were placed in a Coplin jar containing a 2:1 mixture of 2% potassium ferrocyanide and 2% hydrochloric acid for 30 min in a 37°C water bath. The slides were then rinsed with distilled water and counterstained with nuclear fast red (Vector Laboratories) for 20 min.

Macrophage Staining

For indirect immunohistochemistry, 5-µm-thick serial sections were obtained from paraffin embedded tissues. Slides containing tissue sections were heated in a 60°C oven for 1 h to facilitate adherence of paraffin-embedded tissue sections to slides. Deparaffinization and rehydration of slides was achieved by several changes of PRO PAR clearant and progressively decreasing grades of ethanol. Pretreatment of tissues with Protease-K (DAKO) was performed for 5 min. The primary antibody was applied to a section for 30 min at room temperature. The antibody was a muramidase polyclonal against Lysozyme, 1:300 dilution. For immunodetection, a DAKO ENVISION⁺ kit containing an ENVpoly-HRP enzyme labeled polymer conjugated rabbit secondary antibody was used and applied for 30 min, followed by a 5 min buffer wash. The tissue section counterstaining was performed using hematoxylin for 1 min followed by progressive alcoholic dehydration ending with application of a cover slip.

Evaluation of Number of Macrophages in a Tumor Tissue

High resolution, bright field, 40x magnification images of tumor tissue were taken from control, USPUO, SPIO, and MPIO derived tumors. Number of brown (macrophage marker) pixels was counted. Results are average with standard deviation from 15–20 different fields of view from each of five slices for each tumor.

RESULTS

Incubation of Melanoma Cells With Iron Oxide Particles Results in Accumulation of MRI-Detectable Intracellular Iron

Iron oxide particles of different diameters were tested as contrast agents for identification of slowly cycling cells. Human melanoma cells (WM3734, WM3918, WM35, and 1205Lu) were incubated with USPIO, SPIO, and MPIO iron particles. Internalization of iron oxide particles by melanoma cells was detected by Prussian Blue staining. Most (>95%) of tested cells took up all three types of iron oxide particles. Iron concentrations per cell were measured in WM3734 cells by inductively coupled mass spectroscopy (Table 1). Melanoma cells very efficiently

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internalized contrast agents at concentrations that increase with particle size and were sufficient for MRI detection (see next section).

MRI Detection of 1 pg of Iron at Different Resolutions

Measurements of iron content in MPIO particles indicated ~1 pg of iron per particle. To confirm that this amount of iron can be detected by MRI and to demonstrate that the sensitivity depends on the resolution, we performed experiments on phantoms containing ~300 MPIO particles dispersed in 2 mL volume of agar. Three-dimensional gradient echo images of 58, 78, 117, 156, and 234 μm resolution were acquired, and the number of hypointense regions produced by iron particles was counted in the entire phantom (Fig. 1). At 58 μm resolution, we detected 326 dark spots, which is in reasonable agreement with the number of dissolved particles. Decreasing the resolution diminished the number of detectable hypointense regions (Table 2). For images with 80-μm resolution, ~80% of the particles were detectable, whereas at 156 μm resolution (in vivo experiment) 26% of the particles were found. The concentration of iron in a cell after overnight incubation was more than 1 pg of Fe (Table 1), which on the basis of the phantom experiments should suffice for MR detection.

Toxicity of Iron Oxide Particles in Melanoma Cells

Melanoma cells (WM3734, WM739, WM3918, 1205Lu, and WM35) were incubated with SPIO (100 μg/mL) particles overnight and allowed to proliferate for 5 days. No differences were detected in the number of viable iron-labeled and control (unlabeled) cells (Fig. 2a). Also, no differences were observed between the relative number of viable cells labeled with USPIO, SPIO (100 μg/mL), and MPIO (45 μg/mL) as well as unlabeled control cells (WM3734) after 5 days of proliferation (Fig. 2b). The ability of labeled melanoma cells to grow as nonadherent cells was evaluated in a colony formation experiment: Melanoma cells (WM3734, WM3918, WM35, and WM739) were labeled with SPIO (100 μg/mL) and grown in a mixture of soft agar and cell medium. No difference in colony formation was detected between iron-positive and control cells after 14–21 days of proliferation (Fig. 2c,d). The result of these two experiments indicated that the concentrations of iron particles used in this study were not toxic and labeled and unlabeled cells exhibited the same properties (except for magnetization).

Detection of Iron-Retaining Melanoma Cells by Flow Cytometry

To test the hypothesis that slowly cycling cells retain the original concentration of contrast agent, we allowed labeled cells to proliferate for 3 weeks, and the number of MPIO-labeled cells was examined by flow cytometry at 24 h, 11 and 20 days after initial labeling. 100% of the cells were labeled after overnight incubation with contrast agent, and a single peak was detected in the flow cytometry diagram at 24 h (Fig. 3b). Cell division led to dilution of the contrast agent. The formation of an iron-free peak and broadening of the iron-positive peak were evident in the flow diagram at day 11 after labeling (Fig.

**Table 1** Intracellular Iron After Overnight Incubation of Melanoma WM3734 Cells With Different Iron Oxide Particles

<table>
<thead>
<tr>
<th>Size of particles</th>
<th>Iron particles concentration in incubation medium</th>
<th>pg of Fe/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPIO ~800 nm</td>
<td>45 μg/mL</td>
<td>87.9 ± 36.1</td>
</tr>
<tr>
<td>SPIO ~150 nm</td>
<td>100 μg/mL</td>
<td>42.5 ± 4.8</td>
</tr>
<tr>
<td>USPIO ~30 nm</td>
<td>100 μg/mL</td>
<td>14.6 ± 2.7</td>
</tr>
<tr>
<td>Unlabeled cells</td>
<td>0</td>
<td>&lt;0.4</td>
</tr>
</tbody>
</table>

**FIG. 1.** Detection of 1 pg of iron at 9.4 T. Representative 2D slices of 3D gradient echo MR images of an agar block with ~300 resuspended MPIO particles. Images were taken with 58, 78, 117, 158, and 214 μm isotropic resolution. Number of hypointense (arrows) regions were counted in entire phantom (see Table 2). An increase of the special resolution decreases a partial volume averaging and more iron particles were detected.
3c). At day 20, the majority of the cells were negative for iron (Fig. 3d left peak); however, a small but distinct subpopulation of cells retained the contrast agent at the level comparable to freshly labeled cells (Fig. 3d, right peak). In an independent experiment, we estimated a doubling time of about 58 h for the WM3734 cells. For these cells labeled with 87 pg of Fe per cell, seven divisions are required to dilute the iron concentration per cell below 1 pg, requiring ~17 days. These estimates of the iron dilution in labeled cells due to cell division are in good agreement with our flow cytometry data. The same experiment was performed with USPIO labeled cells, and a consistent result was obtained (data not shown).

We examined expression of the biological marker of slowly cycling cells in iron-retaining cells. Cells carrying different amounts of iron oxide particles were analyzed for JARID1B expression by semi-quantitative-PCR. Three cell populations were collected in cell sorting experiments. R3 is a cell population with the highest percentage of iron-retaining cells, R4 is a region with cells carrying intermediate and low concentrations of iron oxide particles, and R5 is a contrast-free cell fraction (Fig. 3f). Expression of JARID1B protein in these three cell populations was monitored by semi-quantitative-PCR.

Table 2

<table>
<thead>
<tr>
<th>Resolution</th>
<th>58 μm</th>
<th>78 μm</th>
<th>117 μm</th>
<th>156 μm</th>
<th>234 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of particles</td>
<td>326</td>
<td>243</td>
<td>185</td>
<td>80</td>
<td>63</td>
</tr>
</tbody>
</table>

3D image of agar block with ~300 MPIO particles were acquired and number of hypointense (see Fig. 2) regions were counted.

FIG. 2. Toxicity test of iron oxide particles on different melanoma cells. a: A percentage of viable cells (WM739, WM3918, 1205Lu, and WM35) after 5 days of proliferation of SPIO labeled (full bar) an unlabeled (empty bar) cells. b: A percentage of viable WM3734 cells labeled with MPIO, SPIO and USPIO after 5 days of proliferation (full bar) an unlabeled (empty bar) cells. Cells were labeled with 45 μg/mL of MPIO and 100 μg/mL of SPIO and USPIO particles, viable cells were counted manually, nonviable cells were excluded based on Trypane Blue stain, and reported results were average of five independent replicates. c: Colony formation of labeled and unlabeled cells. Cells (WM3734 and WM3918) were labeled with 100 μg/mL of SPIO particles and grown in agar/medium mixture for 2–3 weeks. d: Number of colonies counted after 2–3 weeks of cells (WM3734, WM3918, WM35, and WM739) proliferation. These results indicate that the concentration of iron used in this study is safe and can be used for an investigation for slowly cycling cells.
populations is depicted in Fig. 3e. The highest expression was found in the most iron oxide positive cells, which is consistent with our hypothesis that these cells are slowly cycling. The R5 population expresses significantly lower levels of JARID1B ($P < 0.05$).

In Vivo MRI Detection of Iron Retaining Cells

To demonstrate that slowly cycling/iron-retaining cells can be imaged, we labeled WM3734 melanoma cells with iron oxide particles. Two separate subcutaneous injections of iron-labeled and unlabeled (control) cells were performed in NOD/LiScidIL2Rgnull mice. These xenografts were grown till the tumor reach 1 cm$^3$ volume (5–6 weeks), and animals were MR imaged in a 4.7 T magnet. As expected from our flow cytometry studies, during 6-weeks of in vivo cell proliferation (tumor growth), the iron label was diluted out and only slowly cycling cells retained iron. Figure 4a–c depicts an in vivo MR image of a mouse tumor xenograft grown from iron labeled (top) and control/unlabeled cells (left). Hypointense regions can be clearly seen in a tumor derived from USPIO (a), SPIO (b), and MPIO (c) labeled cells. The control tumor (derived from unlabeled cells) did not show a similar pattern and exhibited a uniform signal intensity.

Ex Vivo MRI Detection of Iron Retaining Cells

We conducted several ex vivo experiments to obtain a higher resolution image of iron retaining cells distribution in tumor tissue. Isolated tumor tissue was imaged with 80 µm resolution. Images of tumor tissue grown from iron-labeled cells exhibited different contrast than images of control tumors: more hypointense areas were detected in “iron-positive” tumors (Fig. 4e–g). An increase in the number of hypointense signals correlated with particles size: more hypointense signals were detected in the tumors derived from MPIO labeled cells compared with USPIO labeled cells. High-resolution
images provided increased details of the tumor vasculature structure. Large vessels as well as punctuated regions containing iron-retaining cells were clearly seen in tumors derived from iron labeled cells. The hypointense regions originating from labeled cells were more pronounced in the vicinity of blood vessels. The existence of iron-retaining cells was confirmed by Prussian Blue staining. Figure 4i–k depicts iron-positive cells in tissue grown from labeled cells, whereas the control tumor showed no iron-positive cells (Fig. 4h).

Detection of Iron Positive Macrophages and Iron Retaining Cells

The hypointense signal may have originated not only from iron-retaining melanoma cells but also from macrophages that had taken up secreted iron or iron oxide particles from dead cells. To assess this possibility, tumor sections from the mice injected with USPIO, SPIO, and MPIO labeled cells were double stained with lysozyme, a marker for macrophages, and with Prussian Blue for iron detection. Figure 5a shows cells that were Prussian Blue negative and lysozyme negative cells indicating the presence of iron-retaining melanoma cells. Therefore, the iron oxide particles are distributed among both melanoma cells and macrophages in the tumor. More macrophages were observed in tumor tissue grown from MPIO labeled cells as compared with control, and USPIO as well as SPIO labeled cells (Fig. 6). A strong variation of macrophages distribution within the tumor tissue was observed, which generated a large standard deviation.

DISCUSSION

The primary goal of this study was to develop a noninvasive method for in vivo imaging of slowly cycling cells. We hypothesized that the slowly cycling melanoma cells play a critical role in tumor recurrence and metastatic dissemination. We believe that in vivo imaging of slowly cycling cells can help to delineate the location and distribution of these cells in tumor tissue, which is important for understanding the role of these cells in tumor regrowth. Also, an ability to detect slowly cycling cells in vivo would enable studies the role of these cells in metastatic dissemination. Preclinical determination of
this information is essential to the formulation of effective strategies for the clinical treatment of melanoma.

Iron oxide particles are contrast agents that are widely used for cell tracking by in vivo MRI (13,28,37–41). A key impetus for development of this technique was its application to stem cell therapy. MRI imaging of cells labeled with iron oxide particles was used to track cells grafted for a treatment of brain pathologies (28,29,42), injured spinal cord (43), and implanted pancreatic islets (39). Iron labeled cells can be easily detected in MR images as a signal void that occurs around labeled cells. Contrast generated by labeled cells is stronger at higher concentrations of the contrast agent. To increase the detectability of implanted cells, several attempts have been made to increase the iron concentration per cell: by increasing the iron concentration in the incubation medium (42), by using higher iron content particles (44) and by using electroporation to incorporate higher levels

FIG. 5. Detection of iron-retaining melanoma cells and iron positive microphages in tumor tissue derived from SPIO labeled cells. a: Representative sections showing lysozyme-positive and Prussian blue—negative cells, indicating the presence of reactive macrophages, (b) lysozyme-positive and Prussian blue—positive cells indicating the presence of iron taken by macrophages, and (c) lysozyme-negative and Prussian blue—positive indicating the presence iron-retaining melanoma cells. ×20 Magnification. d–f: Digitally magnify views of the regions indicated by the boxes in (a–c).

FIG. 6. Number of macrophages founded in control, USPIO, SPIO, and MPIO derived tumors. The total number of macrophages increased in the presence of MPIO labeled cells. USPIO and SPIO derived and control tumors showed comparable amount of macrophages. A large standard deviation is due to significant differences in numbers of macrophages found in different parts of a tumor.
of iron per cell (45). To optimize the labeling protocol for MRI detection of slowly cycling cells, we used iron oxide particles of three different dimensions, 30, 150, 860 nm in diameter, assuming that larger particles carry more iron. Overnight incubation of melanoma cells with each of these reagents led to labeling of nearly 100% of the cells. Maximum iron cell loading was achieved with the largest iron particles: ~85 pg of iron per cell was detected after incubation of cells with MPIO (Table 1) and progressively smaller iron content for SPIO (42 pg/cell) and USPIO (14 pg/cell) labeled cells. Accumulation of these amounts of iron per cell after in vitro labeling suggested that detection of a small subpopulation of slowly cycling/iron-retaining cells in tissue was possible.

**Phantom Study**

Because of the small size and low iron content of each individual particle, no one has yet been able to detect a single USPIO or SPIO particle by MRI. However, MRI detection of individual MPIO particles has been reported at 11.7 T (46). Phantom experiments in the present study were performed to demonstrate that these particles can be detected at 9.4 T and to determine the optimal resolution of MR images for detection of labeled cells. MPIO particles used in this experiment were ~0.8 µm in diameter and had a concentration of about 1 pg of iron per MPIO particle as measured by mass spectrometry. Based on experiments with diluted MPIO particles in agar phantoms, this iron concentration was sufficient for MR detection of individual particles at 9.4 T (Fig. 1). Experiments on phantoms demonstrated that for particles with these dimensions and iron content 50 µm resolution at 9.4 T was required for detection of most of the particles. Decreasing the imaging matrix led to a progressive decrease in the number of detected particles, and diminution of the resolution to 150 µm (for in vivo images) led to detection of only 30% of the particles (Table 2). This drastic drop in detectability is most likely due to partial volume averaging effects. It is important to emphasize that 0.8 µm iron particles were distinguishable in images with 50–234 µm pixel dimensions. Such a strong “blooming effect” significantly facilitates the detection of labeled cells but may lead to errors in estimation of the number of labeled cells based on the number and areas of hypointense regions in an image.

**Toxicity of Iron Particles**

High-intracellular iron concentration can lead to cell toxicity. A number of studies were performed to determine the concentration of iron oxide particles required to induce a toxic effect on cells (47–50). A delay in proliferation and some cell death was detected in our experiments with neuronal stem cells after labeling with high concentrations of SPIOs (500 µg/mL) (Ref. 42; Magnitsky, unpublished data). Even though iron toxicity most likely depends on the cell line, a large variety of cells show no adverse effect on exposure to very high iron concentrations (up to 0.5 mg/mL). Most of the toxicity studies reported in the literature were performed on stem cells, whereas we were interested in labeling cancer cells. To verify that labeled cancer cells had the same properties as unlabeled cells, we compared proliferation of five different iron labeled melanomas with proliferation of their unlabeled counterparts. No difference was detected at the concentrations used in our experiments (Fig. 2a,b). Unlike normal epithelial cells, melanoma cells can grow and form colonies without a solid substrate. We detected no difference in colony formation between labeled and unlabeled melanoma cells grown in 3D medium (Fig. 2c,d). These results indicate that the concentration of iron used in this study (100 µg/mL for SPIO, USPIO, and 45 µg/mL for MPIO) was safe and can be used for future investigations.

**Iron Retaining Cells**

Dilution of the contrast agent due to cells division has been recognized as a limitation to many MRI experiments (28,29,40). In the present study, we took advantage of this phenomenon to selectively labeled and image cells with a slow proliferation phenotype.

The relative population of slowly cycling melanoma is small (~1%) and, therefore, hard to detect. Our flow cytometry experiments demonstrated that iron oxide particles can be used for the selection of slowly cycling cells. Most of the cells diluted their intracellular iron content, but a small fraction of slowly cycling melanoma cells retained a detectable concentration of label after 20 days of proliferation (Fig. 3). Semi-quantitative-PCR analysis of several sorted subcell populations showed stepwise correlation between iron-retaining cells and expression of JARID1B, a biomarker of slow cycling cells. Differences between the most iron-positive and iron-free populations were statistically significant. This correlation confirms our hypothesis that iron-retaining cells are slowly cycling.

**MRI Detection of Iron Retaining Cells**

We were able to detect slowly cycling cells in tumor tissue after implantation of labeled cells into immunocompromised mice. Human melanoma cells labeled with USPIO, SPIO, and MPIO and unlabeled cells were grafted into NOD/ScidIL2Rγcnull mice. After 5–6 weeks of in vivo growth, tumors from labeled and unlabeled cells were clearly detectable, and no differences in tumor volume, shape or any other parameters were detected by visual inspection. However, MRI images of tumors grown from iron labeled cells showed very different contrast compared with images of tumors grown from unlabeled cells. We detected hypointense regions in tumor tissue derived from iron labeled cells. We attributed this hypointensity to iron retaining cells and not to necrotic regions, as we did not observe necrosis in the control tumors (in the same animal). Stronger contrast was detected in tumors grown from MPIO labeled cells compared with melanoma tumors derived from SPIO and USPIO labeled cells, which is consistent with our measurement of iron content in labeled cells (Table 1). Control tumors grown from unlabeled cells exhibited uniform signal intensity with no signal voids. Examination of excised tumor tissue by high resolution MRI and
Prussian Blue staining confirmed the presence of iron-retaining cells. We also observed that iron-retaining cells formed clusters and tended to concentrate close to blood vessels. This observation is consistent with previous reports that “cancer stem cells” were primarily found in niches located close to the tumor vasculature. Calabrese et al. (51) showed that endothelial cells interact with tumor cells and secrete factors that maintain these cells in a stem cell-like state.

The proposed method for detection of slowly cycling cells has several weaknesses. First, it has long been noted that iron oxide particles generate negative contrast (loss of signal) rather than image brightening, which can be easier to detect against a dark background. Not all signal voids in tumor tissue detected by in vivo and ex vivo MR imaging are due to slowly cycling/iron-retaining cells. Big vessels and necrotic regions also exhibit negative contrast on T2*-weighted MR imaging. Interpretation of experimental data becomes difficult if imaging experiments are performed at late time points when the tumor has developed a mature vasculature system or produced necrotic regions. We observed hypointense regions in control (noniron containing) tumors when imaging experiments were performed on large tumors (not shown). To overcome this problem, the time at which imaging experiments are performed should be taken into consideration; best results are achieved with small tumors.

Second, at the present time, MR imaging can not differentiate iron positive macrophages from iron-retaining melanoma cells. Our histological staining showed a presence of iron positive macrophages in tumor tissue. An evaluation of histological slides of tumors grown from USPIO, SPIO, and MPIO labeled cells showed an increase of iron positive macrophages in parallel with particle dimensions. The amount of macrophages in control tumor (grown from unlabeled cells) is comparable with an amount of these cells in tumors derived from USPIO and SPIO labeled cells (Fig. 6). Conducting experiments with cells labeled with smaller iron particles will reduce the effect of macrophage “contamination” and should produce a more realistic picture of the slowly cycling/iron-retaining cell distribution.

Macrophages are widely present in melanoma. Distribution of these cells in the tumor tissue is extremely heterogeneous: some regions have many of them, some have practically none. Transfer of intracellular labels from implanted cells to macrophages was found in the studies performed by Dr. Joseph Frank’s group from NIH. In this experiment, human or mouse labeled bone marrow stromal cells imbedded into Matrigel were injected into 129/SvLmJ mice and the amount of labeled positive macrophages were estimated by flow cytometry and CD11 staining. Less than 10% of macrophages were labeled after 1–2 weeks of implantation (52). Our histological data showed that even smaller fraction (~1%) of all macrophages is iron positive. At the same time, the iron retaining cells are also very rare (~1%). We were not able to reliably evaluate a fraction of slow cycling melanoma cells to the total iron positive cells. To accurately estimate the ratios of such small fractions, sophisticated statistical analysis with a large group of animals is required.

Comparison of in vivo and ex vivo MRI images of tumors with iron-retaining cells indicated that MPIO particles produce excessively high contrast, and it is difficult to estimate the fraction of slowly cycling cells in the tissue. Iron-retaining cells labeled with SPIO showed very distinct contrast and probably are ideal for most studies. MRI detection of iron-retaining cells labeled with USPIO was more challenging but definitely possible. The fraction of iron positive macrophages detected in this tumor was lower than in SPIO or MPIO labeled tumors, which makes the use of USPIO particles more attractive. Improvement of the in vivo imaging protocol and optimization of the timing of imaging experiments may facilitate detection of iron retaining cells.

Little is known about slowly cycling cells and at the present time there is no toll to observe these cells in vivo. The proposed method of MR detection opens an opportunity to image these subcell population and address number of important biological question, such as: Are slowly cycling cells randomly distributed in a tumor or are they located in designated areas (niches)? How is the distribution of these cells associated with tumor vasculature? Does metastatic melanoma have more slowly cycling cells than indolent? Do these cells migrate within a tumor? The detection of a migration of slowly cycling cells is especially important, since we believe they are involved into metastatic dissemination of melanoma. Two types of migrations can be distinguished. First is microscopic migration of slowly cycling cells within a primary tumor, and second is macroscopic migration of these cells from primary tumor to distant organs. Iron-labeled cells were detected after trans-cardiac implantation of cancer cells in the rats brains by J.A. Frank group from NIH (ISMRM meeting 2009, abstract #396, also see Refs. 53 and 54), whereas we were able to detect microscopic migration of iron labeled cells in a mouse brain after intraventricular implantation (55). Thus, we believe that detection of both types of migrations is feasible until melanoma sub-cells population can preserve a high concentration of iron particles.

In conclusion, we have shown that MRI can noninvasively detect slowly cycling melanoma cells in mouse tumor xenografts. In vitro incubation of melanoma cells with iron oxide particles results in ~100% cells labeling. After long proliferation of the labeled cells, only slowly cycling cells retain contrast agent and can be distinguished from rapidly proliferating cells. Noninvasive MR imaging can detect the slowly cycling cells in tumor tissue 6 weeks after implantation of labeled cells. Monitoring of the slowly cycling cells in animals should extend our understanding of the role of these cells in tumor recurrence and metastatic dissemination.

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