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The *in vitro* effects of a bimodal contrast agent on cellular functions and relaxometry

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ABSTRACT: The *in vivo* monitoring of cell survival and migration will be essential to the translation of cell-based therapies from the laboratory to clinical studies. The pre-labeling of cells with magnetic resonance imaging (MRI) contrast agents renders them visible *in vivo* for serial cellular imaging. However, little is known about the impact of the presence of these metal particles inside transplanted cells. The use of the bimodal contrast agent GRID made it possible to demonstrate by means of fluorescent microscopy and inductively coupled plasma mass spectrometry (ICP-MS) that, after 16 h of incubation (without the use of a transfection agent), neural stem cells (NSCs) were saturated and no longer incorporated particles. With this maximal uptake, no significant effect on cell viability was observed. However, a significant decrease in proliferation was evident in cells that underwent 24 h of labeling. A significant increase in reactive oxygen species was observed for all GRID labeling, with a very significant increase with 24 h of labeling. GRID labeling did not affect cell motility in comparison with PKH26-labeled NSCs in a glioma-based migration assay and also allowed differentiation into all major cell types of the brain. GRID-labeled cells induced a signal change of 47% on T_2 measurements and allows a detection of cell clusters of ~220 cells/μl. Further *in vivo* testing will be required to ensure that cell labeling with gadolinium-based MRI contrast agents does not impair their ability to repair. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: MRI; contrast agent; proliferation; viability; differentiation; toxicity; relaxometry; cellular MR imaging; GRID; stem cell

INTRODUCTION

The limited capacity of significant repair in the brain is in part explained by the inability of the mature brain to generate sufficient new cellular elements. Although endogenous neurogenesis contributes new neurons to regions of injury, this contribution is often insufficient to promote functional repair (1). The potential therapeutic use of exogenous neural stem cells (NSCs) has generated considerable interest because of the potential to circumvent this limitation. NSCs are defined as multipotent undifferentiated cells that are capable of unlimited self-renewal and differentiation into all major phenotypes constituting the central nervous system. Following transplantation into the damaged brain, NSCs have been shown to possess a tremendous migratory potential to sites of pathological damage in animal models of disease, such as focal ischemia (2,3) or gliomas (4).

This therapeutic approach may prove promising, especially in gliomas where effective delivery of therapeutic agents through the blood stream has proved to be difficult. Current understanding of the processes by which transplanted NSCs target regions of pathology have been investigated in the main using immunohistological methods focusing on the regional distribution of engrafted cells (4–6). The snapshot images available for these single time points are thus only offering an inferential assessment of the migratory ability of transplanted stem cells and consequentially are inadequate to track grafted cells *in vivo*. Future clinical trials will greatly benefit from the development of technologies that allow for the non-invasive *in vivo* monitoring of transplanted cells (3).

Magnetic resonance imaging (MRI) affords the repeated non-invasive assessment of brain anatomy and function *in vivo*. It achieves a high level of spatial resolution that exceeds any radioactive imaging and therefore is the method of choice for detecting small populations of cells. Hence, serial MRI of NSC migration *in vivo* is a non-invasive dynamic method for the monitoring of cell transplants (3,7). However, in order

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to be detectable by MRI, NSCs need to be magnetically labeled with contrast agents prior to implantation (8). There are currently two main groups of paramagnetic contrast agents used for this purpose, iron oxide (Fe) based particles and gadolinium (Gd) based chelates.

Small particles of iron oxide (SPIO) contrast agents produce strong hypointensities (dark spots), known as negative contrast, on T_2- or T_2^*-weighted MR scans and allow the detection of as few as 50–70 cells *in vivo* (7,9). Gd-based chelates are the most commonly used MR contrast agents in clinical practice and have an excellent safety record (10). The potential of Gd-based contrast agents to increase T_1 relaxivity (i.e. create a positive contrast effect) is considered advantageous for tracking NSCs *in vivo* (11–15), since negative contrast effects on T_2-weighted images can lead to a false positive identification of cells in the injection tract (8). Signal attenuation on T_2-weighted images can also be affected by bleeding or air bubbles. By changing the anesthetic gases, it is possible to suppress some of the effects of blood vessels, decreasing the likelihood that negative contrast on T_2^*-weighted images is due to vascular effects rather than contrast agent labeled stem cells (16). Nonetheless, depending on the biophysical environment and the weighting of the image, Gd-based agents can produce signal enhancements (T_1 effect) or signal losses (T_2 effect) on MR images.

Irrespective of the type of contrast agent, it is probable that the inclusion of a contrast agent exerts effects on the cellular properties of NSCs (8). To date, this issue has been poorly addressed. In the present study, therefore, an investigation was made of the effects of the bimodal Gd-based (Gd^{3+}) contrast agent gadolinium rhodamine dextran (GRID) on neural stem cells (NSCs). The bimodality of GRID (detectable by both MRI and fluorescence microscopy) is advantageous as it allows a direct comparison of MR images and histological specimens but also provides the opportunity to study how GRID affects essential cellular functions of NSCs *in vitro*. Preservation of these cellular functions is essential for a successful clinical translation of NSCs as a cell-based therapy. Hence, an optimized labeling protocol for sufficient and efficient *in vitro* labeling of NSCs with GRID was first determined. Essential cellular functions, such as viability and proliferation, were then assessed to determine if labeling with GRID would limit cellular survival or affect the cell cycle. Additionally, an assessment was made of whether GRID incorporation resulted in the production of reactive oxygen species that would indicate if free radicals are formed within the cells that could ultimately lead to cell death.

To determine if GRID labeling would affect the ability of cells to respond to pathology, a migration assay probed their ability to respond to pathological cues secreted from glioma cells, whereas a differentiation assay verified that NSCs retained their multipotency after GRID incorporation. The effect of the biophysical environment on relaxivity was also evaluated by measuring changes in relaxivity of GRID-labeled NSCs (i.e. how does cellular incorporation of the contrast agent change its relaxivity). These assays helped the authors to investigate the effects of GRID on cellular function and the relaxivity of contrast agent labeled NSCs.

MATERIALS AND METHODS

Neural stem cells (NSCs)

Conditionally immortalized murine neural stem cells from the MHP36 line were grown according to previously described standard culturing conditions (17). As described by Sinden *et al.*(17), this cell line was derived from the heterozygotic H-2K^b-tsA58 transgenic mouse's E14 hippocampal proliferative zone anlage. In these cells, the temperature-sensitive mutant of SV40 is brought under the control of the interferon-inducible H-2K^b promoter. MHP36 cells therefore proliferate at 33 °C but cease to proliferate and start to differentiate when cultured under appropriate conditions at 37 °C. MHP36 cells were grown in fibronectin-coated (Sigma, UK) flasks at 33 °C with Dulbecco's modified Eagle medium/F12 (DMEM) (Invitrogen, UK) supplemented with 2 mML-glutamine (Invitrogen, UK), 380 ng/ml L-thyroxine (Sigma, UK), 0.03% bovine serum albumin pathocyte (ICN), 58 ng/ml progesterone (Sigma, UK), 5 μg/ml insulin (Sigma, UK), 15 μg/ml putrescine dihydrocloride (Sigma, UK), 95 μg/ml apo-transferrin (Sigma, UK), 0.5 ng/ml interferon (Peprotech), 4 ng/ml basic fibroblast growth factor (Peprotech), 38 ng/ml sodium selenite (Sigma, UK), 10 units/ml heparin sodium salt and 58 ng/ml triiode-L-thyronine (Sigma, UK). Cultures were fed every 2–3 days. All cells were passaged when 90% confluence was reached (once a week) using Versene (Invitrogen, UK) to harvest the cells.

In vitro labeling of cells

MHP36 cells were labeled *in vitro* by adding GRID to the growth medium. GRID is a bimodal contrast agent with both paramagnetic and fluorescent properties. GRID consists of a dextran polymer backbone, amino-modified $(CH_2)_4$–NH_2 with covalently attached Gd^{3+} chelates and tetramethylrhodamine with a total molecular weight of ~17 kD and a reported T_1 relaxivity (R_1) of 17 $mM^{-1}\,s^{-1}$ at 1.5 T (18,19). GRID was diluted to 1:1 in phosphate-buffered saline (PBS) and stirred for 16 h at room temperature prior to labeling. For the viability, proliferation and fluorescence assays, GRID was added to the media at a 1:40 (45 μM Gd^{3+} and 2.73 μM tetramethylrhodamine per ml of growth medium) concentration for 2, 8, 16 and 24 h. For all other experiments, cells were incubated with GRID for 16 h. Control cells were also labeled with 5 μM PKH26 dye (Sigma, UK) in HBSS for 4 min to serve as a control for migration and differentiation studies (20).

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Cellular uptake and dilution of GRID

To determine the labeling efficiency of GRID, cells were fixed with 4% paraformaldehyde (PFA) (Sigma, UK) prior to the measurement of the relative fluorescence of GRID-labeled cells. The intracellular concentration of Gd per cell was measured using ICP-MS. For this assay, GRID-labeled and control MHP36 cells were counted by means of a hematocytometer before being digested overnight by an equal volume of nitric acid. From the digested sample, 0.25 ml was added to a mixture of 0.05 ml indium (which served as an internal control), 0.3 ml nitric acid and 9.4 ml dH_2O. All samples were run in triplicate. The concentration of Gd per cell was calculated by dividing the total Gd content in the sample divided by the number of cells. The preservation of GRID was tested by growing pre-labeled cells for 1, 3, and 7 days at either a proliferative 33 °C or a non-proliferative 37 °C. All experiments were repeated 3 times.

Viability and proliferation

To investigate cellular uptake of GRID and its effect on the viability and proliferation of MHP36 cells, cells were incubated for 2, 8, 16 and 24 h. After removal of the contrast agent with the growth media, cells were washed with Hank's balanced salt solution (HBSS) (Sigma, UK). Cell viability was then determined by a double-staining method using the nuclear dye Hoechst 33342 to label all cells and fluorescein diacetate (FDA, green fluorescence) to label all viable cells (21–23).

Hoechst labeling stained the nucleus of every fixed cell on the coverslips. Counting of the number of cells per field of view (15 per time point at ×20) made it possible to assess the effect of the labeling procedure on the proliferation of cells (initial seeding density of 30 000 cells per coverslip). Although this is a basic measure of proliferation that accounts for both cell division and cell death, it allows a direct comparison between labeled and unlabeled cells in terms of the total number of cells that are present in the culture dish at a given time. Additionally, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Molecular Probes, UK) was used to measure mitochondrial metabolism associated with proliferating cells. For this, the culture media was replaced with a 100 μl mixture of MTT (pH adjusted to 7.2) and culture media (1:10) for 4 h at 37 °C. Media was aspirated and replaced with 20 μl of 0.25% trypsin. The 96-well plate was then placed on a shaker at a high speed for 5 min at room temperature. A 100 μl mixture of isopropanol and 0.04 N HCl was added to the well and placed back on the high-speed shaker for 15 min to dissolve the formazan. The relative fluorescence indicating the level of mitochondrial metabolism was measured using a 96-well plate fluorescent spectrometer. All measures were taken in triplicate and are expressed in relation to control cells.

Reactive oxygen species (ROS) assay

After three washes of media, GRID-labeled and control cells were incubated for 60 min with (5- and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFCDA) (Invitrogen, UK) diluted in PBS (10 μM). The green fluorescence that is emitted when this compound binds to reactive oxygen species was measured in five separate fields from each of three coverslips.

Intracellular localization of GRID

To investigate the intracellular localization of GRID, GRID-labeled cells were incubated overnight at room temperature with a primary antibody against the lysosome-associated membrane protein 2 (LAMP2, 1:200) (Abcam, UK) in PBS. After removal of the primary antibody, coverslips were incubated with the secondary anti-rat Alexa488 antibody (Molecular Probes, UK).

Migration assay

The influence of GRID on the migratory potential of MHP36 cells was investigated using a microchemotaxis chamber assay. To study the motility of GRID-labeled NSCs, use was made of a rat glioma cell line, CNS-1, and fibroblasts (3T3) as attractants. The CNS-1 rat glioma cell line was developed in inbred Lewis rats as described by Kruse *et al.*(24) and was kindly provided by Dr William Hickey (Dartmouth Medical School, NH, USA). The CNS-1 cells were cultivated at 37 °C in RPM1-1640 medium (Sigma, UK) with 2 mM L-glutamine supplemented with 10% fetal calf serum (Invitrogen, UK) and 1 mg/100 ml antibiotics (Gentamicin; Invitrogen, UK), and cells were passaged twice a week using trypsin (Invitrogen, UK). 3T3 fibroblasts, obtained from the European Collection of Cell Cultures (ECACC, UK), were cultivated at 37 °C in DMEM medium (Sigma, UK) containing 2 mML-glutamine, supplemented with 10% fetal calf serum (Invitrogen, UK). 3T3 cells were passaged at 90% confluence using trypsin.

For the migration assay, 60 000 GRID- or PKH26-labeled MHP36 cells were plated onto the upper layer of fibronectin-coated inserts (Millicell®PCF, 12 mm diameter and with a pore size of 8.0 μm; Millipore, UK). The following chemoattractants were plated onto 24 well plates and allowed to establish for 24 h: CNS-1 cells (4×10^4 cells), 3T3 fibroblasts (4×10^4 cells), CNS-1 conditioned medium (collected from flasks with CNS-1 cells) and unconditioned RPMI-1641 medium. Following establishment of these conditions, the MHP36-filled

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inserts were transferred on top of the wells containing the chemoattractants. The distance between the MHP36 inserts and the chemoattractants was determined by the feet of the inserts. Cells were fixed with 4% PFA after 48 h at 37 °C. Cells that did not migrate from the upper insert were removed using a moist cotton bud in order to ensure that only the migrated cells were counted. The number of cells that migrated from the upper to the lower layer of the insert determined the migration. An inverted microscope (Olympus 1×70) was used for the migration assay. Experiments were repeated 3 times.

Differentiation assay

To investigate the influence of GRID on the multipotency of NSCs, the ability of MHP36 cells to differentiate into neurons, oligodendrocytes and astrocytes was assessed in a co-culture assay (20). Fetal cortical cells were derived from mice embryos (E15) and cultured on poly-D-lysine-coated coverslips. These primary cultures were established for 4 days, after which 5000 PKH26- or GRID-labeled MHP36 were seeded onto the cultures. Co-cultures were maintained at 37 °C with 5% CO_2 for 7–10 days in primary culture growth media consisting of DMEM (Invitrogen) supplemented with 0.03% bovine serum albumin pathocyte, 95 μg/ml apo-transferrin, 15 μg/ml putrescince dihydrochloride, 5 μg/ml insulin, 380 ng/ml L-thyroxine, 320 ng/ml tri-iodo-L-thyronine, 58 ng/ml progesterone, 2 mML-glutamine, 38 ng/ml sodium selenite and 0.5% fetal bovine serum (Invitrogen). Cultures were fixed using 4% PFA for 15 min prior to differentiation analyses by immunocytochemistry. Each experimental condition was repeated 3 times.

Fixed cells were washed 3 times in PBS prior to blocking and permeabilization with 3% normal goat serum (Dako, UK) and 0.1% saponin (Sigma, UK) in PBS. Cells were incubated overnight at 4 °C with primary antibodies appropriately diluted in PBS with 1% serum. To determine differentiation into neuronal cells, mouse anti-NeuN (1:200, marker for neuronal nuclei) (Chemicon, UK) and mouse anti-microtubule-associated protein (MAP2, 1:100, marker for neurons) (Sigma, UK) were used as primary antibodies. Mouse anti-glial fibrillary acidic protein (GFAP, 1:100, astrocyte marker) (Sigma, UK) determined astroglial differentiation, whereas mouse anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, 1:100) (Chemicon, UK) indicated a mature oligodendrocyte phenotype and mouse anti-O4 (1:50) (Chemicon, UK) revealed immature oligodendrocytes. Cells were washed 3 times with PBS before subsequent incubation in the dark for 2 h at room temperature with either goat anti-mouse IgG or goat anti-mouse IgM FITC-conjugated secondary antibodies (1:100) (Chemicon, UK). Following three further washes in PBS, cells were counterstained with Hoechst 33342 (bisbenzimide trihydrochloride) (Sigma, UK) and mounted with Vectashield for fluorescence (Vector Laboratories Inc., CA). Negative controls, i.e. cells labeled not with primary but with secondary antibodies, were performed for testing of unspecific binding of the secondary antibody.

Fluorescence microscopy and image analysis

Cells were examined with a Nikon Eclipse (E600) fluorescence microscope and images captured with an in-built digital camera system. A Leica confocal microscope was used for stacked imaging of labeled cells. The LUCIA image analysis system (LUCIA, version 4.21; Laboratory Imaging Ltd, Prague, Czech Republic) was used for image post-processing, while ImageJ (NIH, USA) was used for relative fluorescence measurements.

MR relaxometry

T_2 and T_1 relaxivity (R_2, $1/T_2$; R_1, $1/T_1$) of pure GRID (in distilled water) and the effect of labeled cells on both T_1- and T_2-weighted MR images were investigated using a 4.7 T Oxford 200/300 MkII (Oxford Instruments, UK) superconducting magnet and VNMR 6.1B (Varian, UK) scanning software. Use was made of a spin echo (SE) T_2-weighted MR sequence with a train of eight echos with increasing echo times (15, 20, 89,159, 228, 297, 367 and 590 ms), a fixed TR of 12.8 s, a field of view (FOV) of 6.5 cm, a matrix of 128×64 and a slice thickness of 2 mm. Based on these images, a T_2 map was generated. For estimation of R_2 of pure GRID, the T_2 relaxation times of three different concentrations of GRID (with 0.18 and 0.018 mM Gd and 0.009 mM Gd^{3+}) were calculated. Doped water, pentahydrated copper sulfate ($CuSO_4$), at three different concentrations (0.96, 2.08 and 3.22 g/l) and with known T_2 relaxation times (100, 150 and 300 ms respectively), was used as a control for the estimation of T_2 relaxation times obtained with the present sequence. The T_1 effect of labeled cells was investigated using a SE sequence ($TR = 200$ and 6000 ms, $TE = 20$ ms) and a modified version of the ratio method for estimation of T_1 relaxation times (25,26).

MHP36 cells were pre-labeled with GRID, washed with HBSS and centrifuged twice in order to remove the excess of the contrast agent. Both labeled and unlabelled cells were then equally distributed at different concentrations in liquid 6% bovine gelatin (Sigma, UK) in separate glass tubes, and the phantoms were allowed to solidify rapidly at 4 °C in order to prevent clumping of cells in the gel. The phantoms were heated up to room temperature (20 °C) prior to scanning. The percentage change in relaxivity was normalized to the unlabelled cells by using the following equation:

$$\% \text{ Change in } R_2 = [(L - U)/U] \times 100\%$$

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where L and U are the T_2 relaxation times for labeled and unlabelled cells respectively. Based on this, an estimate was made of the minimum number of cells needed for a reduction in T_2 relaxation rates and signal attenuation using the general equation for T_2 decay and changes in T_2 relaxation times with specific cell densities.

Statistical analysis

Viability, proliferation, fluorescence and migratory potential were analyzed by one-way or two-way ANOVA using GraphPad Instat 3.0 (Graphpad Inc., San Diego, CA) followed by a Bonferroni post-hoc analysis. Direct comparisons between two conditions were interrogated with independent *T*-tests. Regression analysis of relaxometry data was conducted with SPSS 12.01 (SPSS Inc., Chicago, IL).

RESULTS

Labeling efficiency of GRID

The labeling efficiency of GRID was determined to provide an efficient staining protocol for MHP36 cells that allowed a maximal incorporation of the contrast agent without affecting cellular functions. As GRID is linked to a fluorescent moiety, it is possible to study the labeling efficiency *in vitro* and measure fluorescence as a substitute of contrast agent uptake. MHP36 cells with GRID were incubated for up to 24 h, and the relative fluorescent intensities of the labeled cells at designated time points were evaluated using a fluorescence microscope. GRID was taken up uniformly throughout the cytoplasm of MHP36 cells with a labeling efficiency of 100% [Fig. 1(A)]. A maximum cellular uptake of GRID was achieved following 16 h of incubation

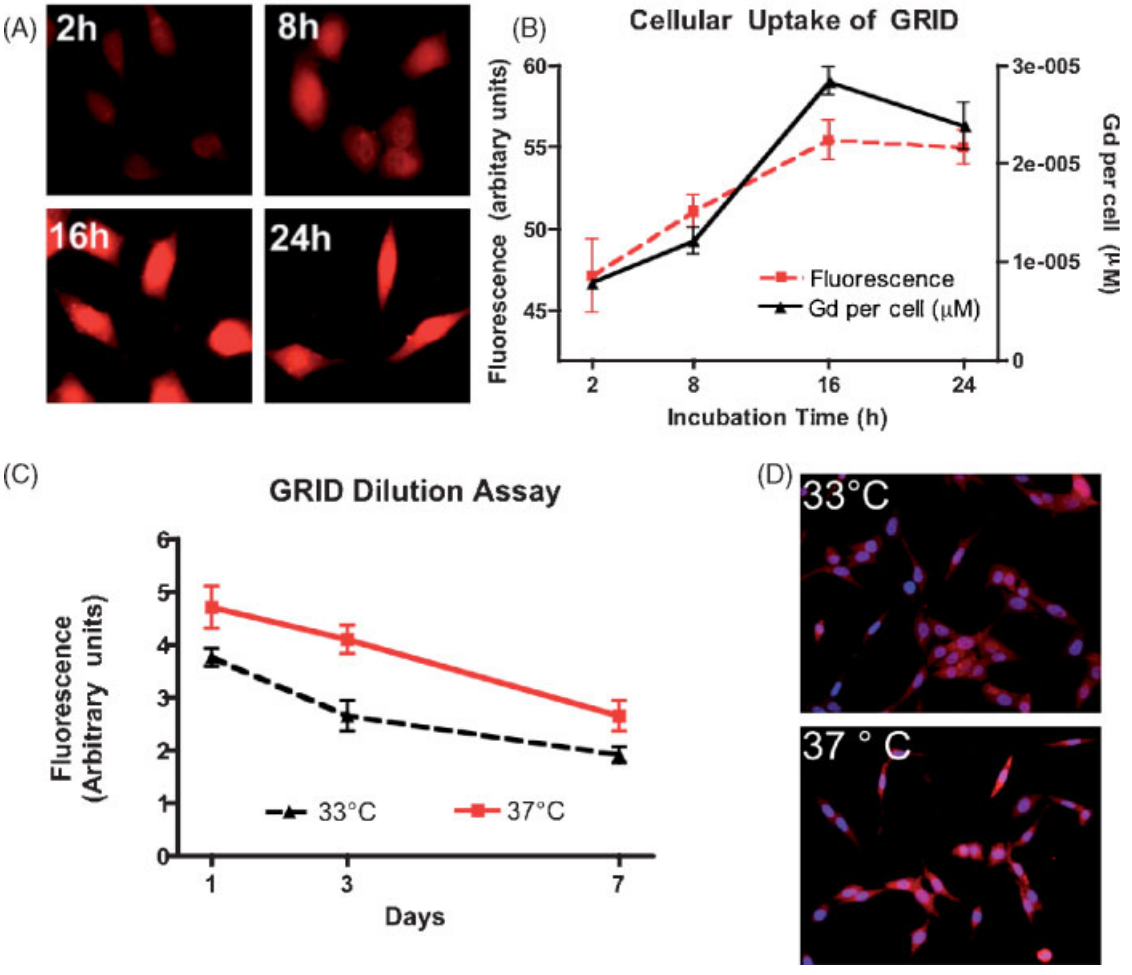

Figure 1. Uptake and stability of GRID. Fluorescence micrographs of MHP36 cells labeled with GRID for 2, 8, 16 and 24 h are depicted in (A), where incubation times of 16 and 24 h give a strong fluorescent signal. Relative measurements of fluorescence and ICP-MS (B) showed a saturation of intracellular uptake of GRID after 16 h. The intracellular stability of GRID was investigated for 7 days, where pre-labeled cells were incubated at the proliferation-permissive temperature of 33 °C or the non-permissive 37 °C (C). After 7 days of *in vitro* proliferation, GRID still allowed a detection of cells similar to the detection of cells under non-proliferative conditions, suggesting that even after five doublings of the cells there was still sufficient GRID inside the cells to allow detection based on fluorescence (D)

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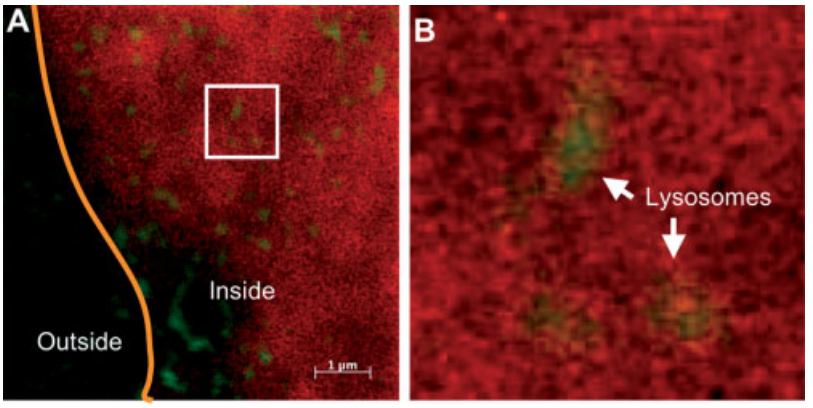

Figure 2. Intracellular distribution of GRID. The immunohistochemical detection of lysosomes made it possible to investigate whether GRID was contained within lysosomes. GRID was equally distributed throughout the cells (A) apart from the nucleus. Some GRID particles were observed inside the lysosomes (in yellow), but a larger quantity of GRID particles (in red) was associated with the surface of lysosomes (in green) (B). Nevertheless, the large majority of GRID did not seem to be contained within lysosomes (B)

($P < 0.05$) with no further increase in fluorescence after 24 h of incubation [Fig. 1(B)].

The same pattern of cellular uptake of GRID was also observed using ICP-MS to determine the intracellular concentration of Gd [Fig. 1(B)]. These results suggest that cells do not incorporate any more GRID after 16 h of incubation and can therefore be considered saturated. Although cells were still detectable by fluorescence microscopy, incubation times shorter than 16 h resulted in significantly lower levels of fluorescence ($P < 0.01$) and intracellular Gd ($P < 0.01$). The intracellular concentration of Gd increased linearly from 2 to 16 h of incubation, doubling the total amount of intracellular Gd.

Cellular preservation of GRID

To allow the continued *in vivo* detection of NSCs by MRI, it is also important to determine the preservation of GRID within cells [Fig. 1(C)]. The present findings indicate an attenuation of fluorescence over time in proliferating cells ($P < 0.01$), but GRID was still detectable using fluorescence microscopy after 7 days [Fig. 1(D)], indicating that GRID is slowly diluted out with proliferation, but that a significant amount of proliferation (>5 doublings) is needed before cells will no longer be detectable. In non-proliferating cells, fluorescence also decreased with time ($P < 0.01$), although this attenuation was less than in proliferating cells ($P < 0.001$). The decrease in proliferation of cells therefore resulted in a better preservation of GRID within each cell. Although cells become non-proliferative under 37 °C and withdrawal of IFN-γ, this downregulation is not immediate but can take up to 3 days and hence results in a decrease in intracellular GRID content. Under both conditions, cells could still be detected after 7 days, demonstrating a good preservation of GRID under both conditions.

Intracellular localization of GRID

Further examination of stacked confocal sections revealed a cellular accumulation of GRID. GRID is widely distributed throughout the cytoplasm and delineates the borders of the cell [Fig. 2(A)]. However, the cell membrane does not contain a greater concentration of GRID compared with the cytoplasm. GRID is equally distributed throughout the cell apart of the cell nucleus (data not shown). Only a minimal amount of GRID is contained within the lysosomes, although a small proportion of particles bound to the outer surface of these vesicles [Fig. 2(B)]. This also suggests that most particles are not sequestered into lysosomes during the cellular uptake process. As cells were stained immediately after labeling, it is also unlikely that GRID was incorporated into lysosomes during the uptake process and then released into the cytoplasm.

Effects on cell viability

The saturation of cells with contrast agent could impact on cellular functions. The impact of cell labeling on viability was therefore investigated. Fluorescein diacetate uptake into all viable cells indicated that GRID labeling did not significantly reduce viability of MHP36 cells compared with non-labeled cells [Fig. 3(A)]. In order to investigate delayed effects of the contrast agents on cellular function, tests of viability were repeated 24 h

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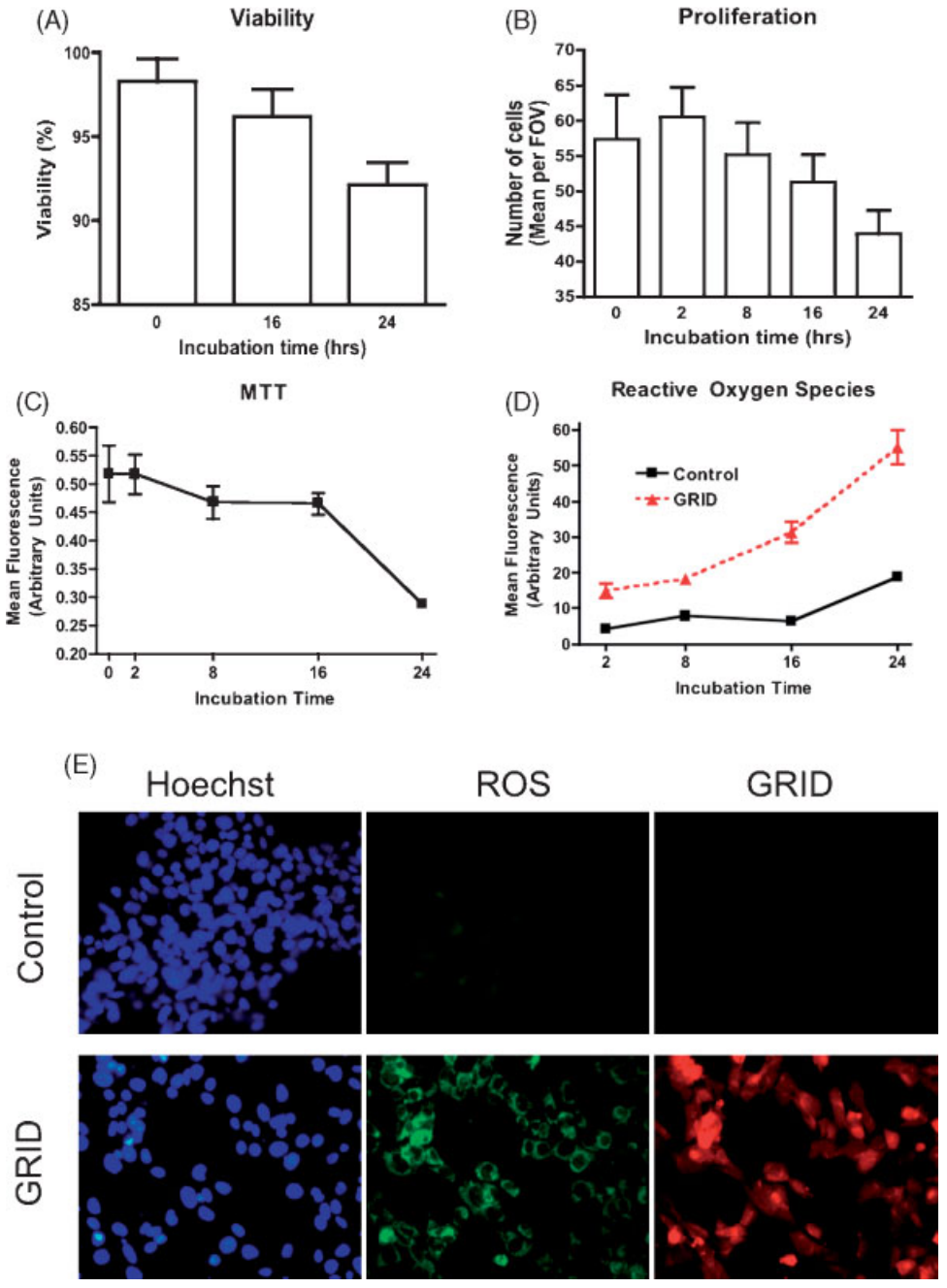

Figure 3. Proliferation and viability of labeled cells. Labeling of MHP36 cells with GRID did not affect cell viability for up to 16 h of incubation, but labeling for 24 h significantly reduced viability by about 6% (A). Cell proliferation was also significantly reduced for 24 h of cell labeling, although a trend to a decrease in proliferation can be observed with increased incubation times (B). A similar trend was observed when measuring mitochondrial activity on the MTT assay (C). In contrast, even the shortest incubation time of 2 h increased the presence of reactive oxygen species (D) which could be detected on the basis of the measurement of green fluorescence. A very dramatic increase in ROS was observed after 24 h of labeling (E), indicating that this duration is very stressful to the cells and potentially could explain the decrease observed in viability and proliferation measures

after removal of the contrast agent from the media. However, the viability of the cells remained high and did not significantly differ from previous measurements (data not shown), implying that cells did not undergo a delayed toxic effect of the contrast agent.

Effects on cell proliferation

Following staining with GRID, the total number of cells per field was determined by the nuclear stain Hoechst 33342 and compared with unlabeled cells. This assay

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provided an evaluation of the impact of the contrast agents on the overall number of MHP36 cells. Although a trend of a reduction in proliferation was observed in GRID-labeled cells with increasing incubation time, no statistically significant effect of GRID on the proliferation of MHP36 cells was observed for up to 8 h of cell labeling [Fig. 3(B)]. However, a significant reduction in proliferation of GRID-labeled cells compared with unlabeled cells (i.e. only 65.3% for 16 h and 60.2% for 24 h compared with controls) was evident ($P < 0.05$), suggesting that GRID might decrease cellular mitosis.

The effect of GRID on cellular mitosis was further assessed with the MTT assay [Fig. 3(C)] which measures the activity of mitochondrial reductase enzymes necessary for cell proliferation. Similarly to the assessment of proliferation by counting the number of cells, the MTT assay indicated that 24 h of incubation with GRID resulted in a decrease in mitochondrial activity to 55.7% of control cells, reflecting a highly significant decrease in the proliferation due to cell labeling ($P < 0.01$). Although both the 8 and 16 h incubation times showed a reduction in mitochondrial activity by 11.2%, this reduction did not reach statistical significance.

Production of reactive oxygen species

Although viability does not appear to be affected by GRID labeling, the ROS assay [Fig. 3(D)] indicated that even after 2 h of GRID labeling, the level of reactive oxygen species was significantly increased ($P < 0.001$). This effect was most dramatic after 24 h of cell labeling ($P < 0.001$) with an almost fourfold increase in ROS-measured fluorescence [Fig. 4(E)] compared with control cells and 2 h of labeling.

Effects on migration

To determine if GRID labeling affects the migratory potential of NSCs, the migration of GRID- and PKH26-labeled cells was compared on various chemoattractant substrates. After 24 h of co-culturing, a significantly increased migration of NSCs (labeled with either GRID or PKH26) towards tumor cells was observed in comparison with fibroblasts ($P < 0.05$), highlighting the migratory potential of these cells and the preservation of this function after GRID labeling (Fig. 4). MHP36 cells showed a greater migratory potential in conditions where cells were used as chemoattractants as compared with no-cell conditions (i.e. unconditioned and conditioned media) ($P < 0.05$), indicating that glioma cells stimulate the migration of MHP36 cells. No significant difference between PKH26- or GRID-labeled cells was observed in any of the four conditions, implying that GRID does not influence the migration of MHP36 cells.

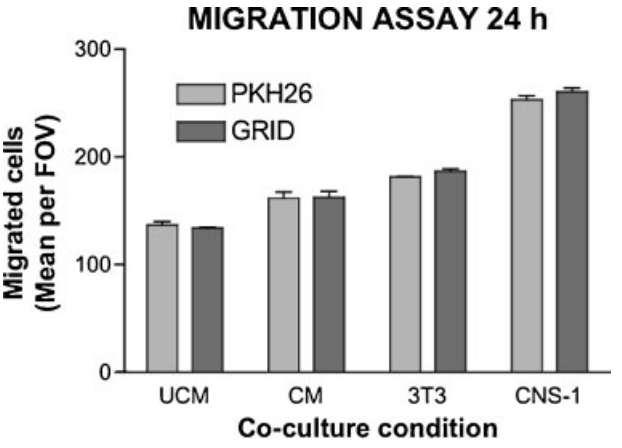

Figure 4. Migration of GRID-labeled NSCs. A migration assay of pre-labeled MHP36 cells was investigated using a micro-chemotaxis chamber. No significant difference in migratory potential could be found between GRID-labeled and PKH26-stained NSCs, indicating that GRID does not exert an inhibitory effect on the motility of MHP36 cells. For both dyes, CNS-1 cells stimulated the migratory activity significantly compared with fibroblasts, suggesting that signals emanating from a pathology induce NSC migration. However, the conditioned media by itself did not induce cell migration. In general, NSCs migrate more in the presence of cells compared with no-cell conditions (i.e. unconditioned and conditioned media)

Influence of GRID on cellular differentiation

MHP36 cells have been well characterized *in vitro* and are known to be multipotent and capable of differentiation into the three major CNS lineages, namely neurons, astrocytes and oligodendrocytes (17,20). To ensure that labeling of MHP36 cells with an MRI contrast agent does not impair their ability for functional repair through appropriate differentiation, it is necessary also to investigate whether cell labeling affects the *in vitro* differentiation potential of MHP36 compared with PKH26. For this purpose, labeled MHP36 cells were cultured with primary cortical cells in a co-culture assay as previously described (20). After 10 days in co-culture, the phenotype of the MHP36 cells was immunocytochemically evaluated. GRID-labeled MHP36 cells differentiated into GFAP-positive astrocytes [Fig. 5(A)] and both immature O4-positive (data not shown) and mature CNPase-positive oligodendrocytes [Fig. 5(C)], and also exhibited neuronal phenotypes as determined by MAP2 (data not shown) and NeuN [Fig. 5(B)]. PKH26-labeled cells exhibited the same differentiation profile (data not shown). These results indicate that GRID-labeled MHP36 cells have the potential to differentiate into all of the three major CNS lineages, and therefore multipotency of NSCs is preserved after GRID labeling.

Relaxometry and *in vitro* detection of labeled cells

As the biophysical environment can change the relaxation properties of Gd-based contrast agents, relaxometry of

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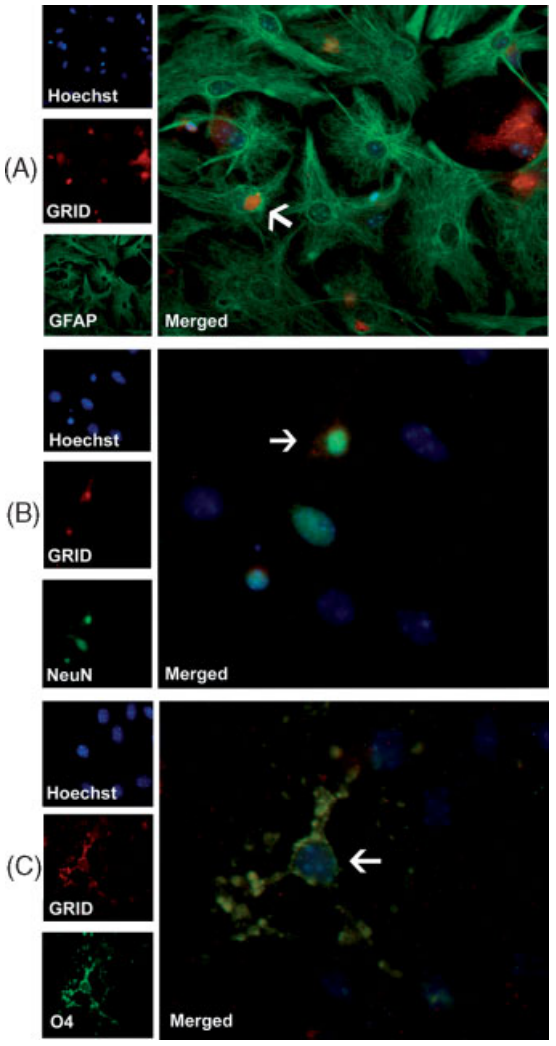

Figure 5. Differentiation of GRID-labeled NSCs. MHP36 cells pre-labeled with GRID were counterstained with the nuclear marker Hoechst 33342 (blue) and phenotypic markers (green) for the astrocytic marker GFAP (A), the neuronal marker NeuN (B) and the immature oligodendrocyte marker O4 (C)

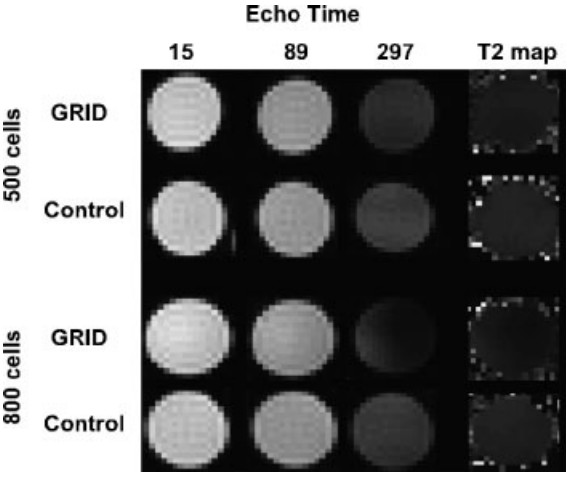

Figure 6. Relaxometry of labeled MHP36 cells. Cells pre-labeled with GRID were embedded in gelatine and scanned using a 4.7 T Varian, where eight echos with increasing echo times (*TE*) were used for calculations of T_2 relaxation times. Here, only three echos and the T_2 map are shown. Depending on the cell concentrations in the vials, GRID-labeled cells shortened T_2 relaxation times by 34 and 47% (for 500 and 800 cells/μl respectively)

pure (in distilled water) and cell-incorporated GRID was undertaken. Using linear regression ($R^2 = 0.919$, $F_{1,7} = 79.5$, $P < 0.001$), the R_1 of GRID was calculated to be $9.9\,\text{mM}^{-1}\,\text{s}^{-1}$ and the R_2 to be $10.3\,\text{mM}^{-1}\,\text{s}^{-1}$. T_2 measurements of doped water revealed T_2 relaxation times of 90, 138 and 287 ms for $CuSO_4$ concentrations of 3.20, 2.08 and 0.96 g/l.

GRID-labeled cells in gelatin induced signal attenuations in T_2-weighted images dependent on the length of echo times (Fig. 6). Labeled cells also induced a reduction in T_2 relaxivity that depended on cellular concentration. At a concentration of 500 and 800 cells/μl, GRID-labeled cells induced a T_2 relaxivity change of 34 and 47% respectively compared with unlabeled cells. To give a 5% relaxivity change with $TE = 67$ ms, the theoretical minimal cell concentration of GRID-labeled cells is therefore calculated to be 220 cells/μl. Interestingly, GRID-labeled cells did not induce a shortening or increase in T_1 relaxation. These results therefore imply that GRID inside NSCs does not induce a T_1 effect, highlighting the importance of the biophysical environment to the induction of a change in relaxivity by contrast agents.

DISCUSSION

Tracing the movement of cells repeatedly in living subjects will allow the non-invasive monitoring of cell-based therapy in clinical settings (27). The Gd-based bimodal contrast agent GRID allows the visualization of NSCs *in vivo* using MRI and *in vitro* using fluorescence microscopy (3,19). Nonetheless, it is essential to determine how contrast agents impact on grafted cells to ensure that labeling of cells does not affect the ability of cells to respond to pathology and promote recovery (8). In the present study it was demonstrated that MHP36 cells can be efficiently labeled within a reasonable time frame (16 h, i.e. overnight) *in vitro* with GRID without the use of transfection agents. Although labeling cells with GRID for up to 8 h seems to have a minimal impact on cellular function, 24 h of cell labeling significantly affected cellular functions, such as proliferation, viability and production of reactive oxygen species (ROS). All incubation times resulted in a significantly increased production of ROS, indicating that the cell labeling affects cellular functions. However, it remains unclear whether an increased production of ROS affects the

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ability of cells to repair, since the migration and differentiation of MHP36 cells, assessed over protracted time points, were not affected by GRID labeling. These results imply that GRID efficiently labels NSCs for the continued monitoring of cellular transplants by MRI, but that extended incubation times will cause detrimental effects on cellular functions, and *in vivo* tests of the ability of cells to repair will be needed to provide a complete assessment of the effect of GRID on cellular functions.

Labeling efficiency and toxicity

GRID provides a very efficient labeling of NSCs. Even after 2 h of *in vitro* labeling without the use of transfection agents, fluorescent microscopy and ICP-MS could detect cellular uptake. Cells were fully saturated with GRID after 16 h of labeling, and further incubation did not result in appreciably higher levels of particle incorporation. The labeling efficiency of MHP36 cells with GRID was 100%. Compared with the efficiency of incorporating iron oxide particles into non-phagocytic cells, even with the use of transfection agents (28,29), GRID-labeling is a very simple and highly efficient labeling procedure.

GRID was preserved inside the cells for at least 7 days under both proliferative and non-proliferative conditions. However, under proliferative conditions, GRID was less preserved than under non-proliferative conditions, although both conditions resulted in a consistent overall decrease in GRID. This decrease in GRID is most likely a consequence of the amount of GRID being halved upon each doubling of the cells. Continued *in vivo* proliferation is therefore likely to hamper cellular MR detection. In contrast, if cells are prevented from proliferating, a better signal can be detected. The conditionally immortalized MHP36 cells downregulate proliferation at brain temperature and differentiate into site-appropriate phenotypes (2). Transplanted MHP36 cells therefore retain GRID better and allow a better MR detection under these conditions (19). Still, transplantation of a tumor cell line that would continuously proliferate is unlikely to be detectable over protracted time points using this method of magnetically labeling cells.

The intracellular uptake of SPIOs through transfection agent (TA) coating of the particles via electrostatic bonds leads to membrane bending that initiates endocytosis (30,31). TA-mediated endocytosis therefore results in a sequestration of the contrast agent into endosomes (9) or lysosomes (32,33). It is shown here by immunohistochemistry that only minimal amounts of GRID are contained within lysosomes, with a larger amount of particles being associated with the outer surface of lysosomes. The vast amount of GRID is located outside lysosomes. The wide distribution and large amount of GRID within the cell could overburden cellular functions. Furthermore, the breaking down of GRID could result in the release of metal particles into the cytoplasm, which might cause free radicals (as suggested by the ROS assay) to be produced. Overburdening of the cell with particles might therefore limit the applicability of this labeling for the purpose of studying how these cells repair damage *in vivo*.

Additionally, the reduction in mitotic processes as compared with PKH26-labeled cells further highlights the potential effects on basic cellular functions if cells are exposed to GRID labeling for 24 h. Although conditionally immortalized cells are not expected to proliferate continuously *in vivo* (34), it is generally anticipated that cells will still initially undergo a few cell divisions prior to committing to a particular cell fate (35,36). A reduction in the ability of cells to proliferate could therefore potentially influence the ability of the grafts to repair damage as fewer cells are produced *in vivo* from the transplant. It is also possible that fewer cells *in vivo* could affect the ability of cells to migrate out from the injection tract and influence differentiation into appropriate phenotypes.

GRID does not impair migration or differentiation

In spite of the effect of GRID on cell proliferation, it did not affect differentiation of transplanted cells. As cell differentiation is an essential criterion for cell replacement therapy, demonstrating that cell labeling does not interfere with this process is a *conditio sine qua non* for the use of this labeling approach for *in vivo* experiments aiming to address brain repair. GRID-labeled MHP36 cells maintained their multipotency *in vitro* after labeling and differentiated into neurons, astrocytes and oligodendrocytes. These results imply that the reduced proliferation observed after GRID labeling does not affect differentiation of GRID-labeled MHP36 cells in animal models of global and focal ischemia (3,19). Nonetheless, it remains unclear if GRID-labeling would preserve differentiation characteristics in other cells, such as mesenchymal stem cells, which have been reported in some cases to show a more restricted differentiation after labeling with iron oxide particles (37). Specifics about the labeling procedure and the contrast agents might account for these 'inhibitory' effects, as other studies (30) found no evidence that ferumoxides interfered with the differentiation of mesenchymal stem cells. More detailed analyses for the different cell types will be needed to determine what aspects of the labeling procedure could influence phenotypic differentiation. In addition to differentiation of cells into site-appropriate phenotypes, MHP36 cells are known to migrate to areas of damage (2,3,38).

It is currently not known if migration is necessary for brain repair, but it is a very attractive property of these cells to target diffuse pathologies, such as invading tumor cells. To investigate whether GRID influences the motility

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of MHP36 cells, the migration of GRID- and PKH26-labeled MHP36 cells was compared using a micro-chemotaxis chamber assay. Glioma cells are known to induce the migration of NSCs both *in vitro* (39) and *in vivo* (4,6,39). The migration of NSCs is thought to be a consequence of the secretion of pathological chemoattractants. Glioma cells from the CNS-1 tumor cell line therefore provided a reliable *in vitro* source of a pathological condition that induces NSC migration. Apart from *in vivo* migration to areas of damage, MHP36 cells are also known to migrate *in vitro* in response to cues, substrates and environments (40). MHP36 cells showed a strong migration towards the glioma cells of the CNS-1 cell line. Interestingly, the effect of glioma cells on the migratory activity was not merely due to released factors, as CNS-1 conditioned media alone was not a potent attractant. However, the released chemoattractants may only have a short half-life, and it is therefore conceivable that they are not active in the conditioned media. Irrespective of the assay conditions, GRID labeling did not affect the ability of cells to migrate in comparison with PKH26 labeling. GRID labeling of stem cells therefore does not affect the ability of cells to migrate in response to pathological cues. This further supports *in vivo* observations of GRID-labeled MHP36 cells migrating towards an ischemic lesion (3) and implies that GRID-labeled stem cells are likely to respond to invading tumor cells *in vivo* as well. In addition to the cellular effects of contrast agents, the *in vivo* monitoring of stem cell therapy also crucially depends on the relaxivity of the contrast agent.

Relaxivity of contrast agent labeled stem cells

The relaxivity of a contrast agent describes its ability to cause a signal change on an MR image. Relaxometry measurements of GRID revealed a T_1 relaxivity (R_1) of $9.9\,\text{mM}^{-1}\,\text{s}^{-1}$ and a T_2 relaxivity (R_2) of $10.3\,\text{mM}^{-1}\,\text{s}^{-1}$ at 4.7 T and previously indicated a R_1 relaxivity of $17\,\text{mM}^{-1}\,\text{s}^{-1}$ at 1.5 T. GRID relaxivity is therefore higher than that of commercially available Gd-based compounds, such as Gd-DTPA ($R_1 = 3.8$, $R_2 = 3.8$ at 1 T) but lower than iron oxide based agents, such as Endorem ($R_1 = 40$, $R_2 = 160\,\text{mM}^{-1}\,\text{s}^{-1}$ at 0.47 T). A theoretical estimation of the lowest number of GRID-labeled cells needed to induce a 5% signal intensity (SI) change on a T_2-weighted image indicated a cell concentration of 220 GRID-labeled cells/μl, i.e. more than 3 times as many cells as reported by Jendelova et al. (9) using Endorem-labeled cells (70 cells/μl). Although GRID-labeled cells induced a shortening of the T_2 relaxation rate by 47% (at a concentration of 800 cells/μl) and would allow the detection of transplanted cells, no T_1 signal change could be observed.

However, the main advantage of the *in vivo* imaging of stem cells with Gd-based agents lies in their potential to induce a positive contrast on T_1-weighted images (11,13–15,41) that could reliably be attributed to transplanted cells. GRID in aqueous media is known to induce a T_1 signal change (18), but upon incorporation into MHP36 cells the T_1 effect is quenched. This difference between GRID in an aqueous solution versus GRID in cells is likely to be due to the intracellular concentration and/or localization of the contrast agent which determines the actual signal attenuation (T_1, T_2 or T_2^* effect) (8). The dominating effect of a contrast agent, be it positive or negative, is not only dependent on particle size, composition and concentration but is also based on the interaction of the contrast agent with water molecules in its vicinity (8). Clustering of GRID in endosomes/lysosomes can lead to a high local concentration of the contrast agent with a restricted access to water molecules and abolish a T_1 effect *in vivo* (15), whereas permeabilization of the cellular compartments by perfusion restores the T_1 effect for *ex vivo* imaging (19). Terreno et al. (15) recently reported that sequestration of a Gd-based agent into the endosomes through pinocytosis quenched the T_1 effect, whereas incorporation of Gd through electroporation preserved this effect.

However, GRID is not predominantly localized inside lysosomes, and it is therefore questionable if the quenching of the T_1 effect here is indeed due to lysosomal or endosomal sequestration. Nevertheless, it is possible that GRID attaches to intracellular elements, such as the surface of lysosomes, and that this impairs its normal relaxivity behaviour. A further possibility is that the GRID content in each cell is sufficiently high after 16 h to negate primary T_1 shortening owing to increasingly dominant T_2 shortening effects. For instance, Aoki *et al.* (14) recently observed a similar effect with manganese, where quantities of >2 mM eliminated the T_1 effect owing to a very significant decrease in T_2. Using alternative uptake mechanisms, such as protamine sulfate (13) or electroporation (15), it might be possible to restore the ability of GRID to produce a T_1 effect upon cell incorporation. Future studies will need to address these issues further in order to provide an optimal protocol for both *in vitro* labeling and long-term T_1-based *in vivo* detection of transplanted cells and an understanding of what factors might quench the T_1 effect.

CONCLUSION

In conclusion, the bimodal contrast agent GRID offers an efficient method for labeling NSCs for cellular MR imaging. It has been reported that GRID does not alter essential cellular functions, such as viability, migration or multipotency of NSCs, while providing enough signal change on T_2-weighted images to allow *in vivo* tracking of transplanted cells. Nonetheless, the reduction

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in proliferation and the increase in ROS after GRID labeling raise concerns. At present, it is unclear what consequences a reduction in proliferation or any other cellular function could have on the ability of cells to repair. Although under ideal conditions no cellular effects should be tolerated for cell therapy, it is unclear if this is possible. In addition to *in vitro* studies, the *in vivo* testing of perturbed cells will be needed to provide better insights as to the tolerable effects cell labeling can exert on cell functions before optimal treatment is affected. The current results highlight the need for detailed *in vitro* and *in vivo* studies to ensure that no ill effects of contrast agent labeling interfere with the success of cell therapy.

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