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Cell labeling with the positive MR contrast agent Gadofluorine M

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Abstract The purpose of this study was to label human monocytes with Gadofluorine M by simple incubation for subsequent cell depiction at 1.5 and 3 T. Gadofluorine M displays a high r_1 relaxivity and is spontaneously phagocytosed by macrophages. Human monocytes were incubated with Gadofluorine M-Cy at varying concentrations and incubation times and underwent MR imaging at 1.5 and 3 T at increasing time intervals after the labeling procedure. R1-relaxation rates and r1 relaxivities of the labeled cells and non-labeled controls were determined. Cellular contrast agent uptake was examined by fluorescence microscopy and quantified by ICP-AES. Efficient cell labeling was achieved after incubation of the cells with 25 mM Gd Gadofluorine M for 12 h, resulting in a maximal uptake of 0.3 fmol Gd/cell without impairment of cell viability. Fluorescence microscopy confirmed internalization of the fluorescent contrast agent by monocytes. The r1 relaxivity of the labeled cells was 137 $mM^{-1}s^{-1}$ at 1.5 T and 80.46 $mM^{-1}s^{-1}$ at 3 T. Imaging studies showed stable labeling for at least 7 days. Human monocytes can be effectively labeled for MR imaging with Gadofluorine M. Potential in vivo cell-tracking applications include targeting of inflammatory processes with Gadofluorine-labeled leukocytes or monitoring of stem cell therapies for the treatment of arthritis.

Keywords Gadofluorine M · Cell labeling · Monocytes · MRI

Introduction

Tissue replacement by stem cell transplants is currently being investigated as a promising approach to treat various diseases, such as myocardial infarction [1], stroke [2], diabetes mellitus [3] and musculoskeletal diseases [4, 5]. Critical questions concerning the fate of the transplanted cells within the host can be answered specifically and non-invasively using cell labeling and cell tracking techniques. Cell labeling with contrast agents for magnetic resonance (MR) imaging provides the ability to monitor the homing and engraftment of the transplanted stem cells non-

invasively with a high anatomical resolution and without irradiation exposure of the host tissue [1–4].

Using MR contrast agents, cells can be either labeled with primarily negatively enhancing T_2 compounds or with primarily positively enhancing T1 compounds. Iron-oxide based T_2 contrast agents offer the advantage of a high sensitivity for cell tracking [6–12]. Different iron oxide compounds, the relatively larger superparamagnetic iron-oxide particles (SPIO) [7, 9–15] and the relatively smaller ultra-small SPIO (USPIO) [7, 9, 11] have been previously applied for the labeling of numerous different cell types, such as various hematopoietic cells [4, 7], including monocytes [11, 14], carcinoma cells [13, 16], embryonic stem cells [3, 18], mesenchymal stem cells [1, 17, 19] and neurologic stem cells [20]. Labeling of human monocytes with iron oxides has been applied in vivo before to monitor neurodegenerative processes in a mouse model [21].

Positive gadolinium (Gd)-based T_1 contrast agents, on the other hand, provide better anatomical detail on T_1-weighted sequences [16, 17]. Especially after interventional procedures for stem cell delivery, the signal loss, induced by iron-oxide-based contrast agents, may be difficult to distinguish from image artifacts, such as susceptibility artifacts from air (e.g., delivered via injection devices or open wounds) or postsurgical iron or hemosiderin deposition [8]. The major drawback of T_1 contrast agents with respect to cell labeling, on the other hand, is their lower sensitivity when compared to iron oxides. This puts high expectations on cell labeling techniques, as higher intracellular concentrations or higher relaxivities must be achieved in order to ensure an in vivo cell detection.

Gadofluorine M is a novel gadolinium-based positive T_1 contrast agent. The chemical structure includes a perfluorinated side chain and a sugar moiety, which leads to formation of micelles in aqueous solutions [22, 23]. The amphiphilic character of this substance may be advantageous for its cellular uptake. Furthermore, Gadofluorine M has a higher T_1 relaxivity compared to conventional small molecular Gd chelates [23]. This may diminish the drawback of the limited sensitivity of Gd-based contrast agents when compared to iron-oxide-based contrast agents. Gadofluorine M has been previously utilized for MR assessment of atherosclerotic plaques [22, 24], MR lymphography in rabbits [23] and nerve degeneration in rats [25]. To our knowledge, this is the first study that deals with cell labeling with this novel contrast agent.

The goal of this study was to establish and optimize cell labeling with Gadofluorine M for the depiction of human monocytes with clinical 1.5- and 3-T MR scanners.

Materials and methods

Contrast agent

Gadofluorine M is an amphiphilic gadolinium chelate complex (Gd-DO3A derivative), developed by the Schering AG (Berlin, Germany). Gadofluorine M contains a perfluorinated side chain, which provides lipophilicity. The prototype complex also includes a sugar moiety, mannose, which increases the hydrophilicity of the compound [23–25]. For our studies, a fluorescent dye, 1,1′-Bis (sulfobutyl)indocarbocyanine-5-carboxylic acid, was covalently attached to the amino group of the lysine backbone of the Gadofluorine M, exactly at the position of mannose. Thus, the sugar moiety (mannose) was replaced by the cyanine dye. The dye has similar properties in terms of hydrophilicity rendering the GadoM-carbocyanine similar to Gadofluorine M itself. In aqueous solutions, Gadofluorine M forms micelles. Due to this self-aggregation, the compound has a long rotational correlation time and, consequently, high proton relaxivities that thus far have only been obtained with macromolecular complexes. Both the original Gadofluorine M and the fluorescent Gadofluorine M-Cy have a molecular weight of 1,528 Da and an r1 relaxivity of 17.4 $mM^{-1}s^{-1}$ in blood at 1.5 T and 37°C. [23]. In addition, Gadofluorine M-Cy exhibited fluorescence with an excitation peak of 521.9 nm and an emission peak of 569.32 nm. Both contrast agents were supplied as an aqueous solution with a concentration of 250 mmol Gd/l.

Cell culture and cell labeling

Human monocytes were obtained from the continuously growing histiocytic lymphoma cell line, U937 [American Type Culture Collection (ATCC), Manassas, VA] and cultured in RPMI 1640 medium supplemented with 10% of fetal bovine serum and 1% of penicillin/streptomycin. U937 monocytes were grown in this medium as a non-adherent suspension culture at 37°C in a humidified 5% CO_2 atmosphere. For the following in vitro studies, the cells were counted in a Neubauer counting chamber and were seeded in six-well plates:

(1) In order to determine the optimal concentration of Gadofluorine M for cell labeling, 5×10^7 monocytes in 5 ml culture medium were incubated with increasing concentrations of 1.25 mM, 12.5 mM and 125 mM Gadofluorine M for 24 h.
(2) To determine the optimal time interval of cell incubation with Gadofluorine M, 5×10^7 monocytes were incubated with 25 mM Gadofluorine M for 1, 2, 4, 6, 12 and 24 h.
(3) To determine the minimal detectable cell number, monocytes labeled with 25 mM Gadofluorine M for

12 h were investigated at decreasing cell quantities of 5.2×10^7, 2.6×10^7, 1.3×10^7, 0.65×10^7 and 0.325×10^7 cells in 0.25 ml Ficoll solution (Sigma-Aldrich, St. Louis, MO). These experiments were performed in triplicate.

(4) To explore the long-term stability of the labeling, 5×10^7 monocytes were incubated with 25 mM Gadofluorine M for 12 h, washed three times and cultured for 1, 3, 5 and 7 days.

After incubation with Gadofluorine M, the cells were washed three times with RPMI 1640 medium by sedimentation (5 min, 210×g, 25°C). Before and after the labeling procedures, the cell viability was determined by trypan blue exclusion.

Cells were transferred to 1.5-ml test tubes for MR imaging. Selected cell samples in 1 ml RPMI 1640 medium were imaged as centrifuged cell pellets in order to provide a visualization of the maximal possible contrast agent effect of the labeled cells. However, these cell pellets were too small for accurate quantitative analyses. Therefore, all cell samples were subsequently imaged dissolved in 0.25 ml Ficoll solution in order to determine signal-to-noise ratios and T_1 relaxation times of the cells. The isotonic Ficoll solution, prepared with a density of 1.07 g/ml, matched the upper density range of the monocytes and provided a preserved viability of the labeled cells during the scanning procedure.

MR imaging

MR imaging of the test tubes was performed using a 3-T MR Scanner and a 1.5-T MR scanner (Signa EXCITE HD 3.0 T and Signa EXCITE HD 1.5 T, GE Medical Systems, Milwaukee, WI) and standard circularly polarized quadrature knee coils (Clinical MR Solutions, Brookfield, WI). Cell samples from experiments (1), (2) and (3) were scanned at 1.5 T and 3 T. Since these experiments showed that the labeled cells exhibited higher R1 values at 1.5 compared to 3 T, subsequent long-term follow-up studies (experiment 4), which focused on the sensitivity of the MR system to detect the Gd-labeled cells, were performed at 1.5 T only. To avoid susceptibility artifacts from the surrounding air in the scans, all samples were placed in a water-containing plastic container and evaluated at room temperature (20°C).

Samples with centrifuged cell pellets were investigated with coronal T_1-weighted spin-echo (SE) 500/16 sequences at 1.5 T and SE 500/15 sequences at 3 T. Samples with cells in Ficoll solution were investigated with axial SE sequences with multiple TR values of 2,000, 1,000, 500 and 250 ms and a TE of 16 or 15 ms at 1.5 T and 3 T, respectively. All sequences were acquired with a field of view (FOV) of 12×12 cm, a matrix of 256×196 pixels, a slice thickness of 5 mm and one acquisition.

MR data analysis

Coronal images of centrifuged cells were analyzed qualitatively by determining any visible contrast agent effect in labeled versus non-labeled cell pellets. For quantitative data analysis, the obtained MR images from cells in Ficoll solution were transferred as DICOM images to a SUN/SPARC workstation (Sun Microsystems, Mountain View, CA) and processed by a self-written IDL program (Interactive Data Language by Research Systems, Boulder, CO). The signal intensity (SI) of each test vial and the background noise in frequency encoding direction adjacent to each vial were measured and expressed as signal-to-noise ratios (SNR=SI/noise). In addition, T_1 maps were calculated from four spin-echo images assuming a mono-exponential signal decay with a fixed TE of 15 ms at 3 T and 16 ms at 1.5 T and variable TR values of 2,000, 1,000, 500 and 250 ms using a nonlinear function least-square curve fitting on a pixel-by-pixel basis. The signal intensity for each pixel as a function of time was expressed as follows: $SI_{\text{pixel xy}}(t)=S_{o(\text{pixel xy})}[1-\exp(-t/T1_{\text{pixel xy}})]$. Care was taken to analyze only data points with signal intensities significantly above the noise level. T_1 relaxation times of cell-bound gadolinium were derived by ROI measurements of the test samples on these maps. R_1 relaxation rates were determined as $1/T_1$. Data from triplicate experiments were displayed as means and standard deviations and tested for significant differences with a Student's t-test. A P-value of less than 0.05 was considered significant. In addition, for calculation of r1 relaxivities of the labeled cells at 1.5 and 3 T, the R_1 relaxation rates were plotted against the Gd concentration in the samples (obtained from spectrometry) using a linear least-squares fit and the KaleidaGraph software program (Synergy Software, Reading, PA). The quality of the fit was determined by the regression coefficient. The r1 relaxivity corresponded to the slope of the curve.

Spectrometry

The Gd concentration within all test samples, including samples of labeled cells and non-labeled controls, were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). The cells in the test samples were dissolved in a microwave (400 W for 55 min) after adding 65% HNO_3 and 30% H_2O_2. For follow-up studies, the culture medium was analyzed for its Gd-content as well in order to identify a potential release of Gd from the cells. The obtained solutions were nebulized into an argon plasma and then analyzed in the spectrometer (IRIS Advantage; FA. Thermo Jarrell Ash Cooperation, MA). A single investigator (HP), who was blinded with respect to the content of the samples and the corresponding MR data, performed these analyses.

Fluorescence microscopy

To determine the amount of cellular uptake of the contrast agent, 5×10^7 monocytes were incubated in Gadofluorine M-Cy (Gadofluorine M linked to the fluorescent molecule Cy3) at a concentration of 25 mM for 12 h and subsequently washed three times with RPMI 1640 media. Then 50 µl of the final solution of the monocytes were spread thinly onto slides and allowed to dry for 5 min at room temperature. After drying, the slides were mounted with Vectashield containing DAPI (Vector Laboratories Inc, Burlingame, CA) and analyzed by confocal microscopy (Zeiss LSM 510 confocal microscope, Thornwood, NY). Images were acquired with a 40× objective lens using two excitation lasers: for DAPI staining a UV laser was used (excitation wavelength 351); for the Cy dye, a HeNe1 laser was used (helium-neon, excitation wavelength 543 nm).

Results

Optimal concentration of Gadofluorine M for cell labeling Qualitative analysis of cell pellets in test tubes showed a readily visible increase in signal intensity of Gadofluorine M-labeled monocytes on T_1-weighted MR images compared to non-labeled controls (Fig. 1). This augmented signal intensity of the labeled cells on T1-weighted MR images increased after incubation in increasing Gadofluorine M concentrations (Figs. 1 and 2). However, though the intracellular Gd uptake increased linearly after cell incubation with increasing Gadofluorine M concentrations (Fig. 2), the MR signal effect, quantified as SNR values, showed only a minor further increase and reached a plateau for cells that were incubated with more than 25 mM Gd/ml (Fig. 2). Thus, the concentration of 25 µM Gd/ml was chosen for subsequent labeling experiments.

Optimal time interval of gadofluorine M incubation Decreasing the incubation interval to less than 24 h resulted in a decreasing Gadofluorine uptake by the cells and, subsequently, decreasing R1 relaxation rates (Fig. 3). Therefore, an incubation period of 12 h was considered optimal for a maximal R1 effect within a minimal time period. The viability of these cells, labeled with 25 mM Gd for 12 h, was 93±7 %, which was not significantly different from unlabeled controls (viability 95±5%, $P>0.05$).

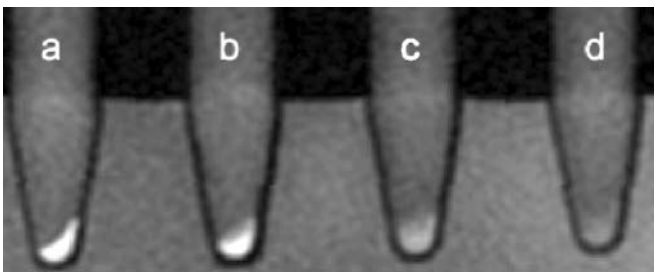

Fig. 1 Representative coronal T1-weighted SE 500/15 MR images of Eppendorf test tubes that contained centrifuged pellets of 5×10^7 monocytes after labeling with Gadofluorine M at concentrations of 125 mM (**a**), 12.5 mM (**b**) and 1.25 mM Gd (**c**). The test tube in (**d**) contains non-labeled control cells

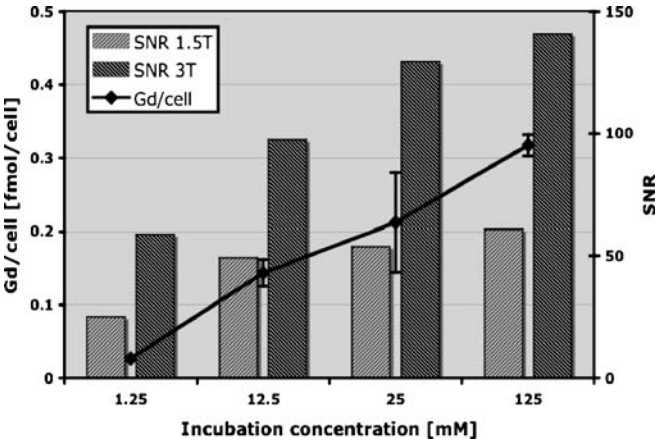

Fig. 2 Intracellular uptake of Gadofluorine M after incubation of 5×10^7 monocytes with increasing contrast agent concentrations for 24 h

Minimal detectable cell number of monocytes Cells labeled with Gadofluorine M were detectable at quantities as low as 5×10^7 cells/25 µl (Figs. 1 and 4), both qualitatively and quantitatively. Fewer labeled cells could not be distinguished from unlabeled control samples ($P>0.05$).

Of note, the R1 effect of the T1-contrast agent, Gadofluorine, was significantly higher at 1.5 T as opposed to 3 T (Figs. 3 and 4). Accordingly, the r1 relaxivity of the labeled cells was 137 $mM^{-1}s^{-1}$ at 1.5 T and 80.5 $mM^{-1}s^{-1}$ at 3 T. These data were not significantly different for

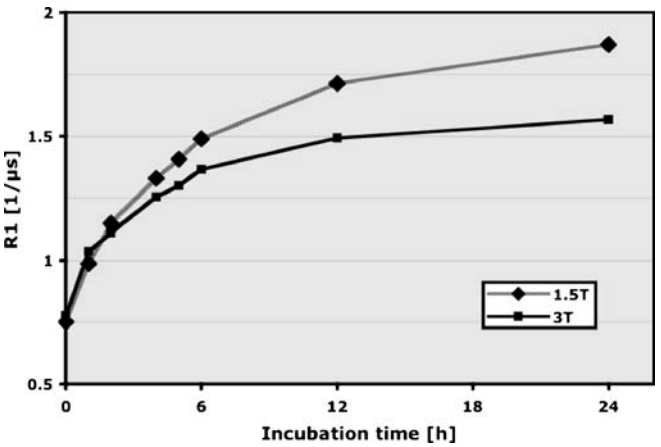

Fig. 3 R_1 relaxation rate of monocytes that were incubated for increasing time intervals with Gadofluorine M at a concentration of 25 mM/ml

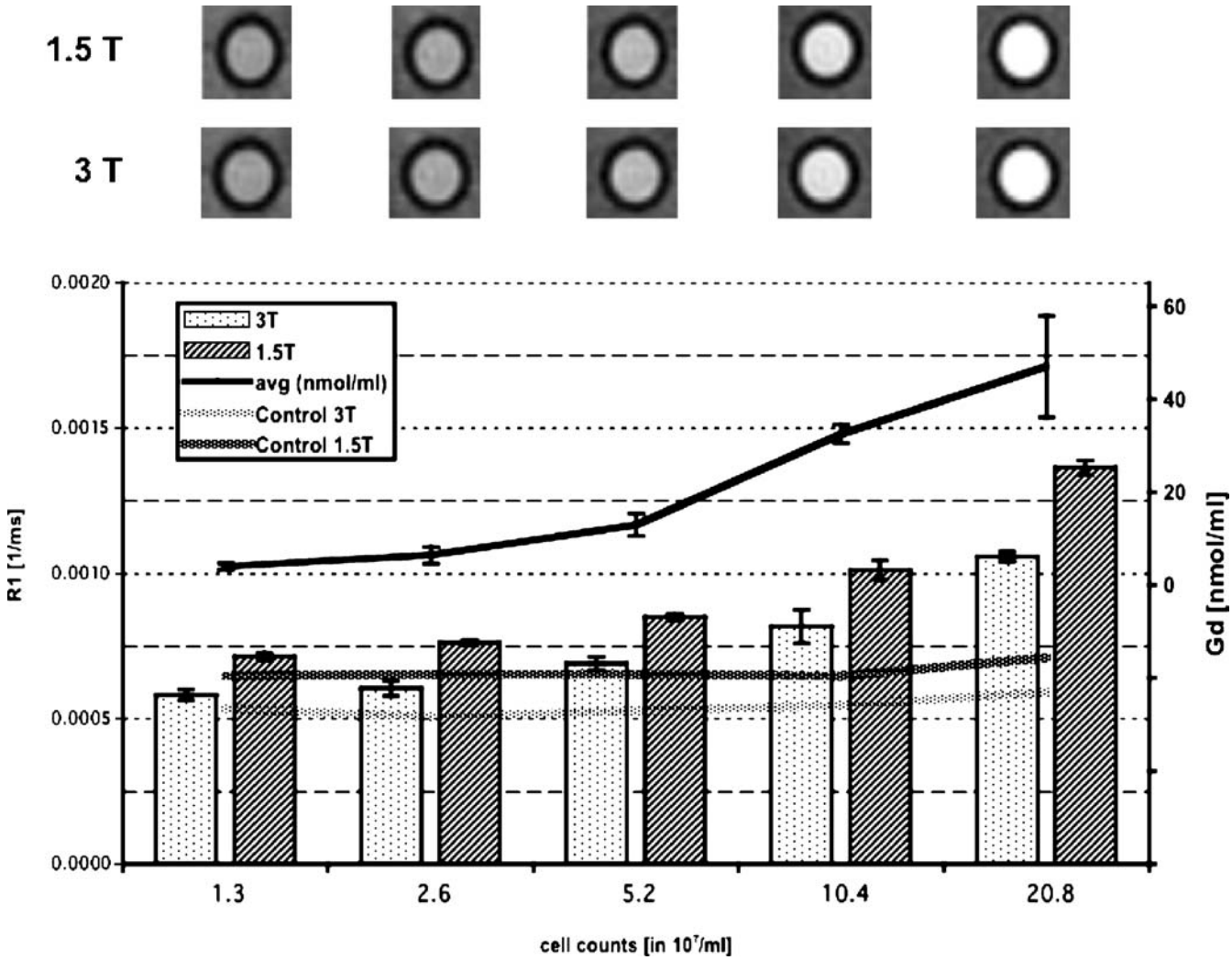

Fig. 4 Dilution series of Gadofluorine M-labeled monocytes at 1.5 and 3 T. The upper part shows axial T1-weighted MR images through Eppendorf test tubes that contained increasing concentrations of Gadofluorine M-labeled monocytes in 0.25 ml Ficoll solution. Controls refer to samples of unlabeled cells. The graph shows the corresponding R1 relaxation rates and Gd contents of the cell samples. The experiment was performed in triplicates; data are displayed as means +/- SD

Gadofluorine M- and Gadofluorine M-Cy-labeled cells ($P>0.05$). However, SNR values of the labeled cells were higher at 3 T compared to 1.5 T (Fig. 2), which is most likely due to decreased noise at 3 T.

Stability of Gadofluorine M labeling over time Monocytes labeled with 25 mM Gadofluorine M for 12 h were washed and subsequently cultured for 1, 3, 5 or 7 days. The labeled cells showed slowly decreasing R_1 relaxation rates over time (Fig. 5). This could be due to a dilution of the contrast agent in proliferating cells, a slow release of the contrast agent from the cells or a combination of both effects. Of note, ICP-AES studies did not identify measurable quantities of Gadofluorine in the cell culture medium during the follow-up study. However, the quantity

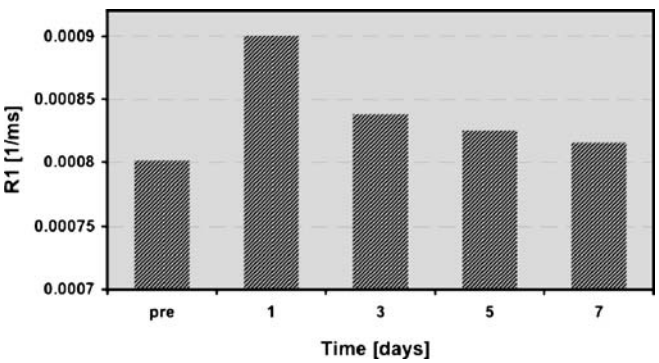

Fig. 5 R1 relaxation rates of the Gadofluorine M-labeled monocytes on follow-up MR studies

of potentially released contrast agent could be below the detection limit of our spectrometry method (100 pg Gd/ml).

Correlation of imaging findings with fluorescence microscopy Uptake of Gadofluorine M-Cy by the monocytes was obvious by confocal microscopy. The contrast agent could be seen as punctate red fluorescence in the cytoplasm of the cells (Fig. 6, **a**–**c**). No contrast agent was found in the nucleus of labeled cells. Non-labeled control cells did not have any red fluorescence (Fig. 6, **d**–**f**).

Discussion

These experiments showed that monocytes incubated ex vivo with Gadofluorine M spontaneously internalized this non-particulate, soluble, small molecular contrast agent. Cellular uptake after incubation with Gadofluorine M was sufficient for detection of the cells by T1-weighted sequences and clinical MR scanners.

The uptake of this contrast agent by monocytes could have occurred via three mechanisms: (1) endocytosis, (2)

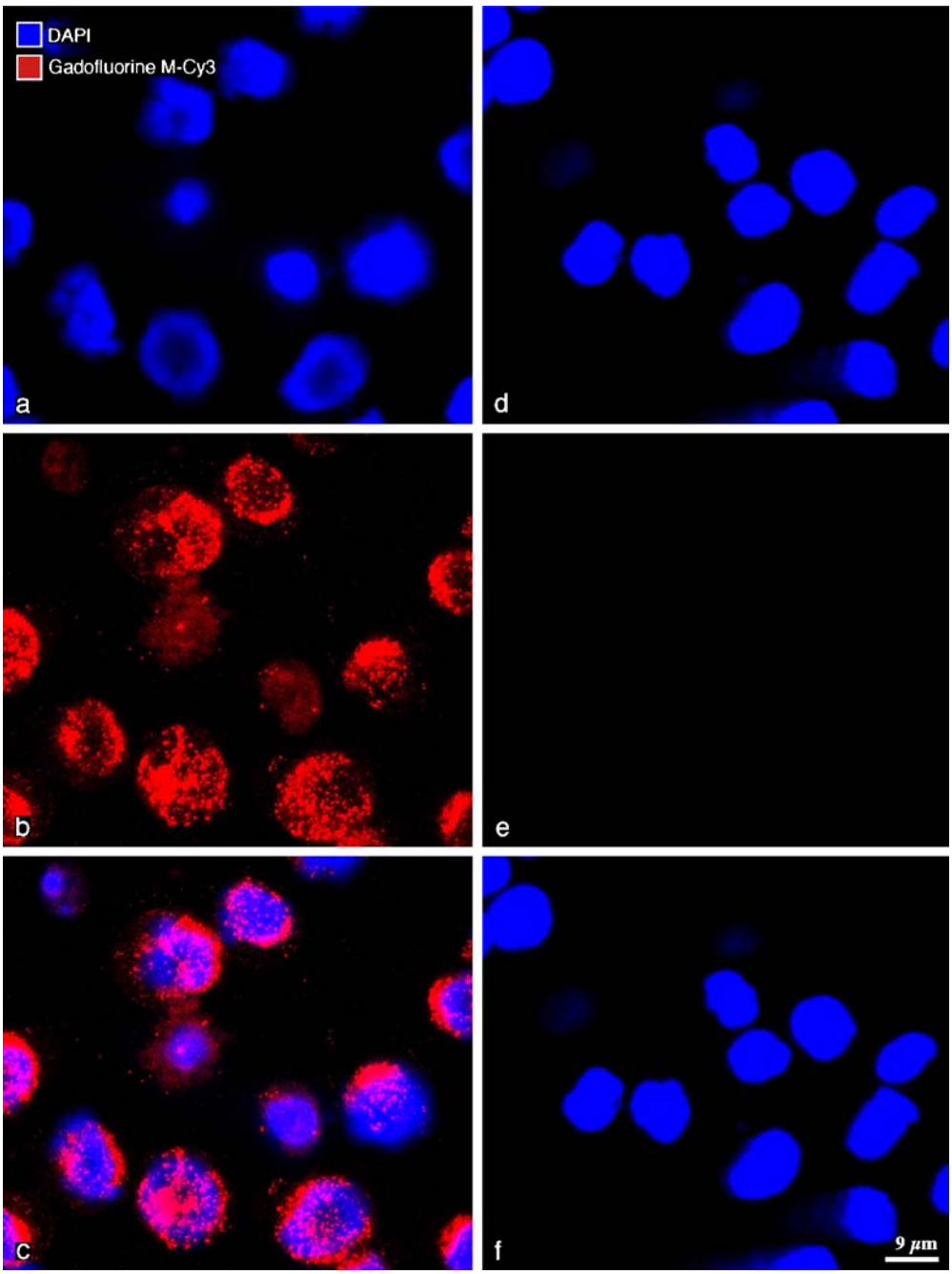

Fig. 6 Confocal microscopic images of U-937-cells. (**a**–**c**) Gadofluorine-Cy labeled cells, (**d**–**f**) non-labeled control cells: **a**, **d** DAPI stained cells; **b**, **e** Gadofluorine fluorescence; **c**, **f** merged images

transmembrane transport or (3) intercalation and cell membrane turnover.

(1) It is well known that monocytes, in general, have phagocytic properties. U937 are expected to have a high rate of macropinocytosis because they are transformed and incubated in serum containing growth factors. In aqueous solutions, such as the culture medium used to incubate cells with the contrast agent, Gadofluorine M forms relatively large micelles or aggregates about 5 nm in diameter, which may be internalized by endocytosis [23]. The micelle formation of Gadofluorine M occurs only in aqueous solutions (in vitro) and not in blood (in vivo), because the perfluorogroup of the contrast agent shows a hydrophobic interaction with hydrophobic components of proteins (mostly albumin) in the blood. To avoid such hydrophobic interactions and to support micelle formation in vitro, which could potentially improve the phagocytosis of the contrast agent, we incubated cells with the contrast agent in serum-free media. For iron-oxide particles, we found a significant, MR-detectable cellular contrast agent uptake for SPIO particles with a diameter of around 100 nm, but not for USPIO particles with a diameter of 20 nm or less [11]. Thus, though we cannot exclude that a component of phagocytosis contributes to the observed cellular Gadofluorine M uptake, it seems that the size of the Gadofluorine M molecule by itself or even the micelles (5 nm) is too small to explain the observed highly efficient cellular uptake of Gadofluorine M in this study. However, macropinocysis or endocytosis with uptake via scavenger receptors may be a possible cellular uptake mechanism for Gadofluorine M.
(2) Specific cell transporters could also facilitate contrast agent internalization. Specific mannose transporters have been found in J774A1 cells (macrophages) [26]. The parental Gadofluorine M includes a mannose molecule, which could have contributed to the observed cellular contrast agent uptake. However, because the measured labeling efficiency was low and the Gadofluorine M-Cy (in which mannose was replaced by the carbocyanine dye) showed a cellular uptake similar to native Gadofluorine M, it seems unlikely that this mechanism is involved.
(3) Another explanation could be that the hydrophobic perfluorinated side chain of the Gadofluorine M molecule intercalates into the lipids of the plasma membrane and subsequently is taken up due to the high rate of plasma membrane turnover in U 937. This property of the molecule also facilitates contrast agent enrichment in lipid-rich atherosclerotic plaques [22, 24]. However, the apparently punctate intracellular localization of Gadofluorine within monocytes on fluorescence microscopy suggests a compartmentalization of the contrast agent to endosomes, lysosomes or other vesicular organelles, which is consistent with an active uptake mechanism, most likely endocytosis.

Gadofluorine M is an amphiphilic molecule and, thus, is able to dissolve in aqueous solutions and human plasma. The amphiphilic structure allows the Gadofluorine M molecule to penetrate phospholipid bilayers [23] and to interact with intracellular water protons, which increases R_1 relaxation times and leads to the observed increased signal on T_1-weighted images. By comparison, conventional Gd-chelates are highly hydrophilic [27] and, thus, are not taken up spontaneously by cells.

Currently established cell labeling techniques are performed with iron-oxide-based contrast agents rather than Gd-based-contrast agents [8, 9, 11, 14, 29]. Iron-oxide particles have been preferred for cell labeling in the past because of their stronger MR signal effect compared to Gd chelates, and, thus, higher sensitivity for detection of the labeled cells. Iron-oxide-based MR contrast agents cause a decreased signal on T_2-weighted images. A considerable disadvantage of this iron oxide-induced T_2 effect, however, is that magnetic field inhomogeneities can be mistaken for a contrast-agent effect. In addition, these contrast agents are metabolized intracellularly, leading to a slowly decreasing contrast agent effect over time [30] and possible toxicity due to non-physiologic intracellular iron concentrations [31].

Positive T_1 contrast agents, like Gadofluorine, have a low sensitivity compared to iron-oxide-based contrast agents. However, T_1 contrast agents have the important advantage of providing a positive contrast on T_1-weighted images, which is less likely to be confused with artifacts due to postoperative local signal voids due to metal or air. Gd-based contrast agents such as Gd-DOTA and Gadophrin-2 have been successfully applied in previous cell labeling and in vivo cell-tracking studies [17, 28]. The Gadofluorine M used in this study displays a higher r1 relaxivity than both Gd-DOTA and Gadophrin-2 and, thus, may compensate to some extent for the inherent lower sensitivity of Gd-based contrast agents compared to iron oxides. Another recently described Gd-based contrast-agent class with high r1 relaxivity is the Gd-fullerenols [16]. However, these agents are complicated and expensive in their synthesis and can be only internalized into cells with the help of additional assisted labeling techniques. Gadofluorine M has the distinct advantage that it can be incorporated into cells by simple incubation procedures that can be applied without specific knowledge in laboratory techniques.

We recognize several limitations of our study: The current studies have been performed in one cell type, human monocytes, to concentrate on variables related to the contrast agent and to exclude variables by different cell types. The most likely mechanism of cellular uptake is macropinocyosis or endocytosis of the Gadofluorine via scavenger receptors. This would suggest that various other cell types could be

labeled by simple incubation with Gadofluorine M as well. In fact, we are currently performing subsequent studies on stem cells.

In addition, we performed limited follow-up studies, up to 7 days after the labeling procedure. During this time interval, we found a slowly decreasing contrast agent effect of the labeled cells, which was due to dilution of the contrast agent in proliferating cells, clearance of the labeled cells, release of the contrast agent from the cells or a combination of these. Of note, ICP-AES studies did not identify measurable quantities of Gadofluorine in the cell culture medium during the follow-up study for 7 days. However, the quantity of potentially released contrast agent could be below the detection limit of our spectrometric method. Further studies are needed to determine if and when the MR signal effects of Gadofluorine M-labeled cells disappear. This will be dependent on the investigated cell type, the initial Gd uptake and the proliferation rate of the investigated cells. Finally, if any cell type could internalize Gadofluorine M spontaneously, this raises the question of whether such a spontaneous internalization could also occur in vivo. According to our results, the cellular Gadofluorine M uptake occurred only after exposure of the cells to very high extracellular Gadofluorine M concentrations, which exceeded by far plasma concentrations after intravenous injection of the contrast agent. We incubated cells with Gadofluorine concentrations of 25 mM Gd, which is about 60-fold higher than the Gadofluorine plasma concentration (0.4 mmol Gd/l, considering an intravascular space of 0.05 l/kg) and 300-fold higher than the Gadofluorine concentration in the extravascular, extracellular space (0.08 mmol Gd/l; considering an extravascular space of 0.25 l/kg) after intravenous injection of Gadofluorine at a dose of 0.02 mmol Gd/kg. Further studies are needed to determine whether Gadofluorine is spontaneously internalized by cells in vivo.

In summary, we have shown that Gadofluorine M is a promising MR contrast agent for labeling monocytes by incubation ex vivo. Potential applications of MR imaging of Gadofluorine M-labeled monocytes include targeting of inflammatory processes or monitoring of stem cell therapies for the treatment of arthritis.

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