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A multi-modality platform to image stem cell graft survival in the naïve and stroke-damaged mouse brain☆

Philipp Boehm-Sturm [a,1], Markus Aswendt [a,1], Anuka Minassian [a], Stefanie Michalk [a], Luam Mengler [a], Joanna Adamczak [a], Laura Mezzanotte [b], Clemens Löwik [b], Mathias Hoehn [a,b,*]

[a] In-Vivo-NMR Laboratory, Max-Planck-Institute for Neurological Research in Cologne, Cologne, Germany
[b] Department of Radiology, Leiden University Medical Center, Leiden, The Netherlands

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ABSTRACT

Neural stem cell implantations have been extensively investigated for treatment of brain diseases such as stroke. In order to follow the localization and functional status of cells after implantation noninvasive imaging is essential. Therefore, we developed a comprehensive multi-modality platform for *in vivo* imaging of graft localization, density, and survival using 19F magnetic resonance imaging in combination with bioluminescence imaging. We quantitatively analyzed cell graft survival over the first 4 weeks after transplantation in both healthy and stroke-damaged mouse brain and correlated our findings of graft vitality with the host innate immune response. The multi-modality imaging platform will help to improve cell therapy also in context other than stroke and to gain indispensable information for clinical translation.

1. Introduction

Transplantation of stem cells is an appealing strategy for a more efficient treatment of stroke since the disease leads to widespread, irreversible cell loss. Neural stem cells (NSCs) are of particular interest for therapy since they can differentiate into all neural cell types without tumorigenic potential. NSCs isolated from embryonic, fetal and adult mammalian tissue and derived from embryonic or induced-pluripotent stem cells have been used in experimental stroke therapy [1,2]. Functional recovery was associated to some extent with NSC migration and integration. Furthermore, modulation of inflammation, angiogenesis, cell death, and plasticity have been described [2]. However, before stem cells can be used for the treatment of patients, the timing, route of delivery, implantation site, and dosage need to be optimized in animal studies [3]. To this end, noninvasive imaging provides information about the spatio-temporal dynamics of cells after transplantation and ideally about their functional state. Thus far, the imaging modality of choice to study cell migration in deep tissues with high resolution is 1H magnetic resonance imaging (MRI) of cells that have been pre-labeled with superparamagnetic iron oxide (SPIO) particles [4]. However, the contrast generated by SPIO-labeled cells is ambiguous against non-homogenous background tissue and cell quantification is extremely difficult. Recently, we and others demonstrated NSC imaging using perfluorocarbon (PFC)-based cell labels, which are detectable via fluorine magnetic resonance imaging (19F-MRI) [5–7]. 19F-MRI provides highly specific information on the localization of cells due to absence of 19F in biological tissue. Moreover, it allows quantification of cell numbers *in vivo* [8]. However, both SPIO- and PFC-based MRI do not provide information on cell viability or functionality. In recent studies, this was overcome by combining SPIO labels with a genetic "label", the luciferase gene [9–11]. Luciferases, in the presence of their substrate, generate a natural form of chemiluminescence called bioluminescence. The luciferase gene can be expressed in mammalian cells, which allows the tracking of genetically modified cells in the living animal quantitatively and noninvasively by bioluminescence imaging (BLI). Since the light is generated from within the living animal and there is no endogenous background signal, BLI is very sensitive. For the experiments reported here, a

* Corresponding author. In-Vivo-NMR Laboratory, Max Planck Institute for Neurological Research, Gleuelerstrasse 50, D-50931 Cologne, Germany. Tel.: +49 221 4726 315/336; fax: +49 221 4726 337.
E-mail address: mathias@nf.mpg.de (M. Hoehn).

[1] These authors contributed equally to this work.

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modified click beetle luciferase (CBG99) was used, which emits light at 543 nm and is particularly favorable for *in vivo* BLI due to its high quantum yield [12].

In previous invasive experiments, a large fraction of cells was reported to die after implantation to the brain [13–15] and the number of surviving cells could not be improved by implanting more cells in a model of stroke [16]. Exact mechanisms and the temporal profile of this cell loss are largely unknown since invasive studies provide only snapshot data from single animals. The rejection by the host innate immune system is thought to be a key player particularly in allogeneic and xenogeneic grafts [17,18]. Interestingly, global cerebral ischemia transiently enhances NSC graft survival in immunocompetent mice [19] indicating that the balance between trophic and inflammatory signals is shifted toward improved cell viability.

Since similar cues associated with neuroinflammation may also be active after focal cerebral ischemia and direct comparison of NSC graft survival in healthy and stroke-damaged tissue is still pending, we developed a quantitative framework to assess transplanted stem cell graft localization and survival *in vivo* using 19F-MRI and BLI. To lower the risk of rejection and in order to focus on the impact of the innate immune system on graft survival we used T-lymphocyte deficient Nu/Nu mice. Using our newly developed 19F-MRI-optical imaging platform in mice that received an NSC implant after focal cerebral ischemia we aimed to i) follow the spatio-temporal dynamics of NSCs in a quantitative manner (19F-MRI) and assess graft survival (BLI), ii) compare survival of NSCs in the naïve and stroke-damaged brain (BLI) and iii) correlate graft survival with the innate immune system response using antibody staining of activated microglia and astrocytes.

2. Materials and methods

2.1. Study design

NMRI-Foxn1nu/Foxn1nu mice (age 8–10 weeks, 25–30 g, male from Janvier, Saint Berthevin Cedex, France) were divided into the following groups: naïve mice that received implantations of multi-labeled NSCs ($n = 7$), pure 19F agent ($n = 3$), nonlabeled NSCs ($n = 12$), or HBSS ($n = 3$) and mice that underwent middle cerebral artery occlusion (MCAO) and received implantation of multi-labeled NSCs ($n = 4$). Animals underwent sequential BLI and MRI up to four weeks. All experiments were conducted according to the guidelines laid out in the German Animal Welfare Act and approved by the local authorities. Numbers represent final numbers after exclusion of animals that did not show significant signal in one of the imaging modalities at the first imaging session. Stroke animals that did not have a lesion on T2-weighted MR images 24 h after surgery or that lost more than 20% bodyweight were also excluded. These criteria were agreed on before the study. One stroke animal was excluded retrospectively since 19F MR images showed strong signal from directly underneath the skin. This indicated a failed transplantation during which cells were pushed back through the injection canal. Replicates are indicated throughout the text and were always true experimental replicates.

2.2. Generation of cell line

Radial glia-like NSCs were derived from the murine embryonic stem cell line CGR8 (generous gift from Prof. A. Sachinidis, Institute for Neurophysiology, University at Cologne, Germany) by adaption of existing protocols [20,21]. The click beetle luciferase CBG99 from pGL3-CBG99 (Promega, Madison, USA) was cloned into the multiple cloning site (MCS) of the lentiviral expression vector pRLL-PGK [22] (kind gift of Prof. Hoeben, Leiden University Medical Center, Leiden, The Netherlands) by the restriction enzymes NheI and XbaI. N2EuroCBG99 cells were generated by lentiviral-vector mediated transduction [22]. For detailed description see Supplementary materials and methods.

A gene marker profile analysis via reverse transcription polymerase chain reaction (RT-PCR) of 20 genes was performed. For the complete protocol and list of primer pairs (Table S1) see Supplementary materials and methods.

2.3. Cell labeling

Cells were seeded (63,000 cells/cm^2) 4 h before labeling on 6-well plates (Greiner Bio-One, Germany). A PFC nanoemulsion with or without fluorescence label (CS-1000 or CS green, Celsense Inc., Pittsburgh, USA) was added at 25 μl/ml for 42 h. A subgroup of 19F-labeled cells received an additional permanent, intracellular fluorescence label (CellTracker Orange – CTO, Life Technologies, Carlsbad, USA) according to the manufacturer's protocol. (Multi-)Labeled cells and unlabeled control cells were harvested with Accutase (PAA Laboratories GmbH, Cölbe, Germany) and centrifuged at 250 × g for 3 min, washed 3× with PBS (PAA) to remove label excess and counted by the trypan blue exclusion method to determine the viable/dead ratio. For further experiments the labeled cells were subsequently prepared in one of the following ways: i) dissolved in HBSS (Life Technologies) for transplantation (150,000 cells/μl), ii) fixed with paraformaldehyde (PFA) to determine 19F/cell with MR spectroscopy as previously described [6], or iii) plated for cell characterization.

2.4. Characterization of multi-labeled NSCs

To assess possible adverse effects of the multi-labeling on cell function, we performed extensive *in vitro* tests of viability, migration, proliferation, differentiation, and luciferase expression on single-labeled (19F or CTO) and multi-labeled (19F and CTO) CBG99 + NSCs. Wildtype (WT) and unlabeled cells served as controls. These assays are described in Supplementary materials and methods.

2.5. Middle cerebral artery occlusion

Focal cerebral ischemia was induced using the filament model as described by Bahmani et al. [23]. Briefly, mice were anesthetized with 1–2% isoflurane in a O2/N2O (30:70%) and received a subcutaneous (s.c.) injection of 4 mg/kg buprenorphin (Temgesic, Merck, Darmstadt, Germany) for analgesia. A silicon rubber-coated filament with a tip diameter of 170 μm (Doccol Corporation, Sharon, MA USA) was used to block the blood flow to the middle cerebral artery (MCA). Animals were allowed to recover during the 30 min occlusion and subsequently reanesthetized to initiate reperfusion by filament removal. The common carotid artery (CCA) was permanently ligated. 24 h after MCAO, animals were scanned with T2-weighted MRI in order to delineate the lesion and to determine stereotactic coordinates for peri-infarct implantations at 48 h post stroke.

2.6. Cell implantation

Implantation as described in detail elsewhere [24] was applied 48 h after MCAO. T2-weighted MRI of the peri-infarct zone 24 h after MCAO was used to determine implantation coordinates. Briefly, mice were anesthetized with Isoflurane in O2:N2O (30:70%), and 4 mg/kg Carprofen (Pfizer, Berlin, Germany) was injected s.c. for analgesia. During surgery, mice remained fixed in a stereotactic frame (Stoelting, Dublin, Ireland). For all naïve and stroke animals, the following coordinates relative to bregma were selected using a stereotactic instrument (Stoeltin): AP +0.5; L +2.0; DV −3.0. 300,000 NSCs were injected into the brain through a Hamilton syringe (26G needle) using a micropump system.

2.7. Bioluminescence imaging

Animals were anesthetized with 2% Isoflurane in 100% O_2, injected with 150 mg/kg D-Luciferin sodium (Synchem, Felsberg, Germany) i.p. and placed on a custom-made holder with two side view mirrors. The time lag between substrate injection and acquisition was recorded for each experiment and used for time-line correction. Bioluminescence data was acquired in list mode for 30 min with the Photon Imager (Biospace Lab, Paris, France) and analyzed by calculations on dynamic time curves with 5 and 60 s temporal resolution [25]. BLI signal change is expressed in % in relation to the data from the first week (day 1 or 7) after transplantation. For multimodal imaging experiments, animals were allowed to recover for at least 6 h before 19F-MRI to ensure wash-out of Isoflurane from the earlier BLI experiment.

2.8. Magnetic resonance imaging

MRI was carried out on a Biospec 11.7 T animal scanner system (Bruker BioSpin, Ettlingen, Germany). For radiofrequency transmission and reception, we used a custom-built, inductively coupled, single-loop surface coil of 20 mm, tunable from 470 MHz for 19F up to 500 MHz for 1H. Anesthesia was initiated using Ketamine/Xylazine (100 mg kg^{-1}/10 mg kg^{-1} i.p.) and prolonged after 40 min by Ketamine (25 mg kg^{-1} s.c. every 15 min). Respiration rate was monitored using a pressure sensitive pad placed under the thorax, body temperature was maintained at 37 °C with an in-house feedback-controlled system, and 100% oxygen was delivered through a nose cone. Animals were fixed with ear bars in standard animal holders (Bruker BioSpin). Anatomical 1H MRI was performed with a turbo spin echo sequence (repetition time/effective echo time = 2.2 s/42.8 ms, 8 echoes per excitation, number of averages = 4, 20 consecutive, coronal, 0.5 mm thick slices, field of view = 2.28 * 1.92 cm^2, 192 * 128 matrix, acquisition time = 2:21 min, bandwidth = 50 kHz). 19F images were acquired with the same sequence and matching geometry but slightly different parameters: effective echo time = 10.5 ms, number of averages = 256, 10 consecutive, 1 mm thick slices, 72 * 48 matrix, BW = 15 kHz, TA = 56:19 min). The imaging session did not exceed 1.5 h. Total integrated 19F signal-to-noise ratio (SNR) of each graft was analyzed as described previously [6]. To study NSC migration, images were registered with an affine transformation to a template mouse brain (average of high resolution T2-weighted MR images from 6 Nu/Nu mice) using FMRIB Software Library (http://www.fmrib.ox.ac.uk/fsl/).