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A chronic 1 year assessment of MRI contrast agent-labelled neural stem cell transplants in stroke

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Abstract

Non-invasive identification of transplanted neural stem cells in vivo by pre-labelling with contrast agents may play an important role in the translation of cell therapy to the clinic. Understanding the impact of these labels on the cells' ability to repair is therefore vital. In rats with middle cerebral artery occlusion (MCAo), a model of stroke, the transhemispheric migration of MHP36 cells labelled with the bimodal contrast agent GRID was detected on magnetic resonance images (MRI) up to 4 weeks following transplantation. However, compared to MHP36 cells labelled with the red fluorescent dye PKH26, GRID-labelled transplants did not significantly improve behaviour, and performance was akin to non-treated animals. Likewise, the evolution of anatomical damage as assessed by serial, T₂-weighted MRI over 1 year indicated that GRID-labelled transplants resulted in a slight increase in lesion size compared to MCAo-only animals, whereas the same, PKH26-labelled cells significantly decreased lesion size by 35%. Although GRID labelling allows the in vivo identification of transplanted cells up to 1 month after transplantation, it is likely that some is gradually degraded inside cells. The translation of cellular imaging therefore does not only require the in vitro assessment of contrast agents on cellular functions, but also requires the chronic, in vivo assessment of the label on the stem cells' ability to repair in preclinical models of neurological disease.

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Conflict of interest statement

None of the authors has a conflict of interest that is relevant to this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroimage.2008.06.017.

Keywords

MCAo; Neural stem cells; Migration; Contrast agent; Cellular imaging; Neural transplants; MRI; Stroke; Gadolinium

Introduction

Transplantation of neural stem cells to recover lost functions after stroke is a promising new treatment approach. Although cell differentiation and integration are thought to underlie functional recovery, the specific neural substrates of repair after stroke damage remain unknown (Bliss et al., 2007). To ensure optimisation of the therapeutic effects of cell transplants, it is therefore crucial to uncover these mechanisms to permit a successful clinical implementation (Bjorklund and Lindvall, 2000).

To understand the dynamic processes involved in stem cell-mediated brain repair, neuroimaging can help to elucidate anatomical and functional changes in relation to behaviour (Modo, 2006). However, to identify the location of transplanted stem cells in vivo and to follow their migration, it is currently necessary to pre-label these cells in vitro with MRI contrast agents (Modo et al., 2005). In stroke-lesioned brains, this approach allows the in vivo tracking of the migration of stem cells from remote regions to areas of damage (Guzman et al., 2007; Hoehn et al., 2002; Modo et al., 2004a; Zhang et al., 2003). Nonetheless, at present it remains unclear how long these cells could be tracked in vivo or if the contrast agent would affect the repair mechanism. The use of a bimodal contrast agent, such as GRID, affords the direct study of cellular uptake in vitro and co-localisation with phenotypic markers to determine how contrast agents affect cellular function (Brekke et al., 2007a; Modo et al., 2002a). Although labelling of cells with GRID does not influence in vitro and in vivo differentiation or migration (Brekke et al., 2007a; Brekke et al., 2007b; Modo et al., 2002a), it is unclear if contrast agent labelling could affect the cells' ability to promote repair based on mechanisms that cannot be tested in vitro.

MRI allows the serial assessment of the same subject over protracted time periods and is therefore ideally suited for the long-term follow-up of therapeutic effects. To monitor the downstream anatomical effects of stem cell transplantation, a volumetric measurement of the evolution of neuroanatomical structures and the lesion can help to pinpoint areas of change that are relevant to the recovery of particular impairments (Roberts et al., 2006). In animal models, MRI can therefore help to determine what changes can be expected from stem cell therapy upon translation into human patients (Modo, 2006; Modo et al., 2004b).

Therefore we have investigated (1) how long GRID-labelled cells were detectable by MRI for in vivo monitoring, (2) if GRID labelling affects the cells' ability to repair and (3) the anatomical substrates underlying neural stem cell-mediated repair of stroke damage.

Methods

Animals

Sprague–Dawley rats (Charles River, UK) were acclimatised for at least a week prior to surgery. All procedures were in accordance with the UK Animals (Scientific) Procedures Act 1986 and the ethical review process of Queen Mary and Westfield College, University of London.

Middle cerebral artery occlusion

Animals between 280–330 g were either allocated for sham or 60 min of right transient middle cerebral artery occlusion (MCAo) surgery (Modo et al., 2002c). Briefly, animals were anaesthetised with isoflurane (4% induction, 2% maintenance) in a mixture of O₂ and N₂O (30:70). Temporary ligatures were placed on the ipsilateral external and common carotid to stop the flow of blood to the internal carotid artery. The tip of the thread was advanced 18–20 mm from the cervical carotid bifurcation or until reaching resistance from the ostium of the middle cerebral artery in the circle of Willis. Following occlusion, animals were tested for spontaneous circling and forelimb flexion (Modo et al., 2000). Occluded animals were re-anaesthetised and the thread was removed.

Preparation of neural stem cells

MHP36 cells—Conditionally immortalised neural stem cells from the Maudsley Hippocampal Clone 36 (MHP36) cell line are derived from the neuroepithelium of the H-2K^b-tsA58 immortomouse at embryonic day 14 (Sinden et al., 1997). These cells proliferate at 33 °C providing an ample source of cells for transplantation, but cease proliferation at 37 °C. In the damaged CNS, MHP36 cells show site-appropriate phenotypic differentiation (Modo et al., 2002c) and are therefore ideal candidates for brain repair (Gray et al., 2000).

GRID labelling—The Gadolinium-Rhodamine Dextran (GRID) conjugate, consisting of a dextran polymer (total molecular weight ~ 16.6 kD) (Huber et al., 1998) and multiply labelled with gadolinium (Gd-DTPA) and rhodamine, was diluted (1:1) with distilled H₂O at least 24 h before cell labelling. Addition of GRID to the growth medium resulted in a final concentration of 45 µM of Gd³⁺ and 2.73 µM of tetramethylrhodamine (chelated in one molecule) per ml of media (Modo et al., 2002a).

For cell labelling, MHP36 cells were grown in proliferative conditions with GRID added to the media. After 8 h of incubation with GRID, the medium was discarded and cells were removed from the flask by adding Hank's Balanced Salt Solution (HBSS, Gibco) without Ca²⁺ and Mg²⁺. The suspension was centrifuged (1500 rpm for 5 min) and cells were re-suspended for transplantation (25000 cells/µl) with 1 mM *N*-Acetyl-L-Cysteine (NAC, Sigma) in Hank's Balanced Salt Solution (HBSS, Gibco). Previous studies have demonstrated that the effects of 8 h GRID labelling are negligible on in vitro assays, although effects on toxicity, proliferation and reactive oxygen species were observed after longer labelling times (>16 h) (Brekke et al., 2007a).

PKH26 labelling—MHP36 cells (passage 42) were removed from the flask by adding Hank's Balanced Salt Solution (HBSS, Gibco) without Ca^{2+} and Mg^{2+} . In suspension, cells were pre-labelled by incubation for 4 min with a 5 μM solution of the fluorescent cell membrane marker PKH26 (Sigma, UK) in HBSS. After 3 washes, cells were re-suspended (25,000 cells/ μl) with 1 mM *N*-Acetyl-L-Cysteine (NAC, Sigma, UK) in Hank's Balanced Salt Solution (HBSS, Gibco, UK) for transplantation.

Grafting

Two weeks following middle cerebral artery occlusion, animals were transplanted in the left (contralateral) hemisphere to probe their migration towards the ischaemic lesion in the right (ipsilateral) hemisphere and its effect on behavioural recovery. Animals either underwent implantation of MHP36 cells labelled with GRID ($n = 8$) or PKH26 ($n = 8$). Normal controls ($n = 8$) and MCAo-only ($n = 8$) animals served as comparison groups.

For transplantation, anaesthesia was induced by intraperitoneal (i.p.) injection of a mixture of 0.1 mg medetomidine hydrochloride (Dormitor, C-Vet Products), 5.5 mg ketamine hydrochloride (Ketaset, C-Vet Products), in 0.1 ml of 0.9% saline (Baxter) per 100 g bodyweight. Animals were placed in a stereotaxic frame and an incision was made exposing bregma. Two burr holes were drilled contralateral (Site 1. AP = + 0.7, L = 2, V = - 5.5/- 2, Site 2. AP = - 0.3, L = 3, V = -5.5/- 2.5) to the ischaemic lesion containing 2 deposits each. Each deposit consisted of 2 μl at a speed of 1 $\mu\text{l}/\text{min}$. The syringe was left in place for 2 min after injection to allow dispersion of the cells. A total of 2×10^5 cells in a total volume of 8 μl were injected per animal. After grafting, animals were injected with 0.1 mg atipamezole hydrochloride (Anti-Sedan, C-Vet Products).

Immunosuppression

On the day of grafting, animals were immunosuppressed by a subcutaneous injection of cyclosporine A (10 mg/kg body weight, Novartis, Switzerland) in saline (Baxter, UK). This immunosuppressive regime was repeated on alternate days for 14 days after grafting. Although we previously demonstrated that MHP36 cells show equivalent graft survival in immunocompetent and immunosuppressed animals (Modo et al., 2002b), immunosuppression was induced here to replicate the experimental conditions from previous behavioural studies (Modo et al., 2002c; Veizovic et al., 2001).

Behavioural assessment

The bilateral asymmetry test (BAT) is a test of tactile extinction probing sensory neglect (Modo et al., 2000). For the BAT, two strips of brown sticky tape (Dudley, UK) of equal size (6 cm long, 0.5–0.8 cm wide) were applied with equal pressure to the saphenous part of the forepaws in a pseudo-random fashion. Time to remove the labels completely from each forepaw was recorded to provide a measure of asymmetry between both paws, but also provided a total time to remove both labels. Each trial lasted for a maximum of 180 s. For each assessment, animals were given four consecutive trials.

Magnetic resonance imaging

MRI hardware—¹H MRI was performed using a 4.7 T superconducting magnet (Oxford Instruments, UK) interfaced to a UNITY Inova-200 MR imaging console (Varian Inc., USA) and a 63 mm internal diameter quadrature birdcage radiofrequency coil (Varian Inc., USA).

In vivo MRI—Animals were anaesthetised for the duration of the imaging session using Isoflurane (4% induction, 2% maintenance) in a mixture of O₂:N₂O (30:70). MR image acquisition consisted of a conventional spin echo sequence (TR = 4000 ms, TE = 64 ms, number of averages = 4, matrix = 128 × 128, FOV = 3 cm × 3 cm, number of slices = 30, slice thickness = 0.350 mm, in plane resolution = 0.234 mm × 0.234 mm). Animals were scanned 2 days pre-grafting followed by a series of post-grafting scans at 1, 4, 12, 26, 39 and 52 weeks. Lesion volume was then defined as the total number of voxels in the ipsilateral hemisphere that were >1 standard deviation above the mean of the ROI in the contralateral hemisphere excluding the ventricles. To determine structural volumes, ROIs corresponding to these structures were drawn manually using the DISPIM (Dave Plummer, UCL) program.

Ex vivo MRI—To provide high-resolution MR images of the stroke brain, 3 animals per group were perfused (see below) after 1 year with Gd-DTPA-BMA (Omniscan, Amersham Health) added to the 4% paraformaldehyde (PFA) perfusate (see below) to increase the signal on T₂-weighted MR images (TR = 4000 ms, TE = 64 ms, 16 averages, FOV = 2.5 × 2.5 cm, matrix size = 256 × 256, in plane resolution = 0.098 × 0.098 mm, slice thickness = 0.4 mm, 50 coronal slices).

Histological assessment

After the final scanning session, animals were given a lethal dose of pentobarbitone (120 mg/kg i.p.) and transcardially perfused with cold 0.9% saline solution followed by 4% PFA in 0.1 M phosphate buffered saline (PBS). Coronal 50 µm thick sections were cut on a cryostat (Leica, Germany) at −24 °C and retained free-floating in 30% sucrose. Primary antibodies used for identification of neurons (monoclonal mouse anti-NeuN, 1:1000, Chemicon, UK) or astrocytes (polyclonal rabbit anti-GFAP, 1:3000, DAKO, UK) were applied overnight before being incubated with a green fluorescent cross-adsorbed goat anti-mouse or goat anti-rabbit Alexa 488 (1: 500, Molecular Probes, UK) for 45 min. Negative controls omitted the primary antibody and were incubated only in PBS. Sections were washed 3 × 5 min in PBS before being mounted using Vectashield for immunofluorescence (Vector, UK).

As part of the normal ageing process, the cytoplasm of cells in the CNS accumulates the autofluorescent pigment lipofuscin. Treatment with Sudan black can quench this autofluorescence (Schnell et al., 1999). For this, sections were incubated with a 1.5% solution of Sudan black (Sigma, UK) in 70% industrially methylated spirit (IMS, VWR, UK) for 20 min before being washed 3 × 5 min in PBS.

Statistical analyses

To determine group differences and their temporal evolution, repeated measures or two-way ANOVAs were calculated using SPSS for Mac (v.11) followed by Bonferroni post-hoc testing to control for multiple comparisons. A linear multiple regression analysis with a stepwise entering of independent variables was conducted to determine how particular measures would predict behavioural outcome. For this, behavioural outcome measures were separately entered as the dependent variable with all anatomical measures as the independent measures.

Results

Behavioural recovery

Animals with unilateral stroke damage exhibit sensorimotor neglect. This can be assessed by means of the bilateral asymmetry test (Fig. 1). Prior to transplantation, i.e. 2 weeks following ischaemia, all ischaemic animals showed a severe impairment in removing the sticky tape from their affected left paw ($F = 9.518$, $P < .001$). MHP36 cells labelled with PKH26 resolved this impairment between 4 and 12 weeks post-grafting. By 12 weeks, there was no longer a significant difference between MHP36 + PKH26 grafted and control rats indicating that these transplanted subjects recovered from their sensorimotor deficits. However, at this time point MHP36 cells labelled with the bimodal MRI contrast agent GRID did not resolve the forepaw bias ($P < .01$) and performed akin to MCAo-only animals. GRID labelling of MHP36 cells therefore interfered with stem cell-induced recovery on this task. Nevertheless, by 39 weeks (i.e. 9 months), the difference in removal between the left and right forepaw for both GRID-MHP36 transplanted and MCAo-only animals was no longer significantly different from controls or from MHP36+PKH26 subjects.

Although the difference in removal of the tape between both forepaws indicates somatosensory neglect, the total time to remove the tape from both forepaws reflects the strategy as to how animals solve this task (Fig. 1). Upon first exposure to this test, all animals perform equally slowly. However, as the animals learn to remove this tape, they develop strategies as to how best to remove the aversive stimuli. Animals with neglect typically solve this task in a serial fashion. First, they remove the tape from the unaffected paw (ipsilateral to the lesioned hemisphere) that is the main aversive stimulus. Once this has been removed, the stimulus that is perceived as less aversive on the affected forepaw is removed. In contrast, control animals are typically slower in removing both sticky tapes. This is due to switching back and forth between both paws in their removal strategy as they perceive both stimuli as equally aversive ($F = 3.255$, $P < .05$). MCAo-only animals therefore solve this task significantly faster than controls ($P < .05$). Again, MHP36 cells labelled with PKH26 performed as controls, whereas GRID-labelled cells were not significantly different from controls or MCAo-only animals at any time point.

Anatomical repair

To probe anatomical changes underlying the behavioural recovery, serial MRI scans were acquired at the same time points as behavioural testing to provide a concomitant assessment. Typically, prior to transplantation, the ischaemic area was characterised by hyperintense

regions in the striatum and the overlying cortex (Fig. 2A). However, in 37.5% (3 of 8) of animals only striatal lesions were observed. Inside the hyperintense region ascribed as lesion, some tissue could still be observed up to 6 weeks following ischaemia (i.e. 4 weeks post-grafting) that gradually developed into a cavity characterised by a region of T₂-weighted hyperintensity.

Transplanted cells labelled with PKH26 were not detected on MR images, whereas GRID-labelled cells afforded the in vivo visualisation of the location and migration of transplanted cells (Fig. 2B). GRID-labelled cells clearly delineated the injection tract, although by 1 week following grafting 2 out of 8 animals (25%) showed a hyperintensity surrounding this area. This hyperintense area reflects an implantation-induced edema on T₂-weighted MR images. This edema, however, disappeared by 1 month and the injection tract became clearly visible again, but could no longer be detected later than 3 months after implantation (data not shown). Within 1 week following implantation, GRID-labelled cells were already visible in the peri-infarct area, but formed a line that did not correspond to the edge of the hyperintense lesion area (Fig. 2B). By 1 month following engraftment, the edge of the lesion receded to the area delineated by the GRID-labelled cells indicating that the “non-invaded” tissue was lost between 1 week and 1 month. GRID-labelled cells therefore defined the edge of the eventual lesion cavity. Depending on the location of the lesion, GRID-labelled cells showed a different localisation pattern (Fig. 2C). In animals with both a striatal and cortical lesion, GRID-labelled cells can only be found at the edge of the lesion in a single line, whereas in striatal only lesions, grafted cells surrounded the lesion. In areas undergoing transformation from normointense to hyperintense in striatal lesions, GRID-labelled cells integrated into the tissue and temporarily reduced the development of hyperintensity in this area.

To evaluate how GRID- or PKH26-labelled cells affected the development of the lesion, serial MR images were quantified to provide measures of the lesion and its effect on different anatomical structures, such as the cortex and striatum. The advantage of serial MR imaging is that the initial, pre-transplant scan can be used as a baseline for each animal to evaluate how stem cell transplants alter particular measures over time. Notably, this approach can also be used to assess to what degree brain volume changes over time. Controls showed a linear increase of 13% in brain volume over 52 weeks (Fig. 3). This is significantly higher than MCAo-only or transplanted animals ($F = 14.342$, $P < .001$). MCAo-only animals also exhibited a greater increase in brain volume compared to transplanted animals ($P < .05$), but there was no difference between the two groups of transplanted animals. This significant difference in the rate of brain growth can be attributed to the 2nd surgical intervention in transplanted animals that delayed brain growth in comparison to the other two groups.

Lesion volume as identified by T₂-hyperintensity increased 25% in MCAo animals by 4 weeks post-grafting (i.e. 6 weeks post-ischaemia) in relation to pre-transplant lesion volume ($F = 21.078$, $P < .001$). From thereon, lesion volume did not change indicating full cavitation (Fig. 3). Animals with PKH26-labelled transplants also showed an increase in lesion volume of 18% by 4 weeks after grafting suggesting that transplants achieved some attenuation of lesion growth during the consolidation period of the cavity. Little change in lesion volume

was observed between 4 and 12 weeks, but from thereon a steady decrease in lesion volume was observed with a total of 11% absolute reduction in lesion volume in comparison to pre-transplant volumes. In comparison to MCAo-only animals, a significant reduction of 35% in lesion volume was observed after 1 year with PKH26-labelled cells ($P < .01$). In contrast, GRID-labelled cells did not significantly impact on reducing the ischaemic lesion volume and even exhibited a slightly increased lesion in comparison to MCAo-only animals. The lack of anatomical repair by GRID-labelled cells indicates that marking of MHP36 cells with this bimodal contrast agent interfered with the stem cell-induced recovery process.

A similar pattern of group differences can be observed on other anatomical measures (Fig. 3), such as a change in the ipsilateral striatum ($F = 11.824$, $P < .01$) where the PKH26-labelled cells limited the extent of degeneration and significantly restored striatal tissue ($P < .05$). However, there was still a significant lack of striatal tissue compared to non-lesioned controls ($P < .05$). This graft effect translated to an increase of 8% more tissue when compared to the pre-transplant striatum and 21% more than in MCAo only. GRID-labelled cells did not impact on total lesion volume. The contralateral striatum, in contrast, initially showed a significant difference between controls and all MCAo animals ($F = 10.305$, $P < .001$), but gradually this difference disappeared for the animals transplanted with MHP36 cells. GRID-labelled cells reduced the development of the contralateral striatum ($P < .01$). Contralateral, striatal volumes in MCAo-only animals gradually increased and were no longer significantly different from controls at the final time point.

Although more variable than the striatum, the cortex on the ipsilateral side of the infarct is also very significantly reduced in MCAo animals compared to controls ($F = 16.256$, $P < .01$). The damage in both PKH26-MHP36 transplanted and MCAo-only animals did not evolve significantly over the whole year and remained smaller compared to controls ($P < .01$). GRID-labelled cells increased cortical damage in relation to both PKH26-MHP36 transplanted and MCAo-only animals by 4 weeks ($P < .01$) and only saw a moderate improvement over time. The contralateral cortex for control and PKH26-MHP36 grafted animals evolved in a similar fashion and did not reflect the progressive decrease that was observed for MCAo-only and GRID-MHP36 grafted rats ($F = 9.041$, $P < .001$).

As both ipsilateral and contralateral regions might undergo different changes that could affect behaviour (such as rotation due to unilateral striatal damage), it is also important to investigate if transplanted cells affect the asymmetry between homologous brain areas. A reduction in striatal asymmetry was evident for PKH26-MHP36 transplanted animals ($F = 25.070$, $P < .001$), but neither GRID-MHP36 nor MCAo-only animals reduced this difference over time. Interestingly, no significant improvement in cortical asymmetry ($F = 5.963$, $P < .05$) was observed in any of the animals.

Neuroanatomical basis of behavioural recovery

The significance of anatomical changes in relation to behavioural improvements were further explored by using a stepwise multiple regression analysis to provide an indication as to which structures, or combination of these, would best predict performance on the bilateral asymmetry test. Overall, the total amount of time animals required to remove the sticky tape from their forepaws was best predicted ($F = 10.797$, $P < .001$) by a combination of the ratio

between the ipsilateral and contralateral striatum ($P < .001$) and the evolution of the contralateral striatum ($P < .001$) accounting for 41% of the variance. In contrast, 62% of the variance in forepaw bias is predicted ($F = 20.634$, $P < .001$) by lesion volume ($P < .001$) and size of the ipsilateral striatum ($P < .001$). The lack of cortical involvement in these predictions is likely to reflect the inconsistency of cortical damage in our model. Even animals with a preserved primary somatosensory cortex will show a forepaw bias due to the lack of signal transmission through the damaged striatum and therefore the degree of striatal damage is more important here to predict behavioural impairment.

Ex vivo magnetic resonance imaging

Ex vivo magnetic resonance imaging was conducted to provide higher spatial resolution 3 dimensional images of the damaged brain 1 year after grafting. In vivo, a compromise in spatial resolution and image quality needs to be traded against the speed of acquisition and can reduce the detection of smaller clusters of GRID-labelled cells. Although in vivo the injection tract was no longer visible beyond 3 months, ex vivo MRI images delineated a hypointense area reflecting the injection site (Fig. 4A). The higher spatial resolution and averaging can lessen the partial volume effects that limit in vivo detection of these cells. PKH26-MHP36 transplanted animals did not show a clear hypointensity in these areas on ex vivo scans possibly indicating that it is GRID rather than the glial scarring that is the underlying cause of this hypointensity. A hypointense area surrounding the lesion cavity can also be observed in PKH26-MHP36 transplanted animals indicating that the signal decrease is not merely due to the GRID in transplanted cells.

Interestingly, in the lesion cavity small fibrous structures can be observed (Fig. 4B). In vivo these fibrous elements cannot be detected possibly due to the lower spatial resolution. It is possible that these fibres represent elements of glial scarring, but the location and the trajectory rather suggests blood vessels, such as the tributaries of the MCA. Notably, the MCA is reperfused in this model after 60 min of occlusion and hence could continue to supply blood to this area despite the dramatic loss of neuropil.

Histological assessment

To investigate if GRID persisted for 1 year inside cells, animals were perfused and underwent histological analyses. After 1 year of grafting, GRID-labelled cells were still found to delineate the injection tract and the lateral ventricle (Fig. 5A). The transplanted cells with both GRID and PKH26 labels populated the horn of the ventricles and integrated into the subventricular zone. Likewise, along the glial scar of the ischaemic lesion, cells containing red fluorescence were observed (Fig. 5B). However, insufficient GRID cells could be found here that would induce a significant hypointensity on the ex vivo MRI scans. Some of these cells co-localised with GFAP, a marker of astrocytes, whereas others co-localised with NeuN, a marker for neurons revealing that cells differentiated into appropriate mature phenotypes (Suppl. Fig. 1). A similar distribution of cells labelled with PKH26 could be observed with a stronger red fluorescent signal in the peri-lesion area (data not shown).

Although the peri-lesion and injection tract are known to contain transplanted cells at protracted time points, areas outside of these regions are more difficult to interpret since

control animals showed a strong red autofluorescent punctate staining in the cortex similar to the appearance of GRID and PKH26. This autofluorescence is mainly associated with neuronal cells in the cortex and is known as neuronal lipofuscin (Figs. 5C–E). Sudan black can taint this autofluorescence, but unfortunately we also noted here that it quenches the fluorescence of GRID and PKH26. This endogenous autofluorescence prevents a reliable identification of transplanted cells in the cortex. Using the dense red fluorescence of PKH26 and GRID, it is therefore only possible to confirm that transplanted cells are still present in the injection tract, surrounding the ventricles and the lesion cavity. Consequently, outside of these areas, it is impossible to reliably investigate the survival of transplanted cells with our current approach.

Discussion

Cellular MR imaging allows the in vivo tracking of transplanted stem cells migrating to the site of lesion (Hoehn et al., 2002; Modo et al., 2004a; Zhang et al., 2003). However, to date it was unclear over what time periods transplanted cells could be detected in vivo using the gadolinium-based bimodal contrast agent GRID. The use of serial imaging over 1 year indicated that GRID-labelled cells could be detected in the peri-infarct area up to 1 month post-grafting, but no longer provided a sufficient signal for in vivo detection by 3 months, although the injection tract was still visible at this time. After 3 months, GRID labelling no longer afforded any in vivo detection, although ex vivo MRI demonstrated that GRID-labelled cells could still produce hypointense signals in the injection tract 1 year following engraftment.

Chronic effects of GRID labelling

Whilst GRID labelling allows the in vivo visualisation of cell migration and location, the contrast agent interfered with the MHP36 cells' ability to reduce a forepaw bias which is indicative of somatosensory neglect. Nevertheless, GRID-labelled cells were spatially distributed in a comparable fashion to MHP36 cells labelled with PKH26 (Modo et al., 2002a; Modo et al., 2004a) and also differentiate into appropriate phenotypes in vitro (Brekke et al., 2007a) and in vivo (Modo et al., 2002a). A lack of functional repair is therefore unlikely to be attributed to inappropriate migration, distribution or differentiation. It is, however, possible that a small fraction of the contrast agent preparation has a lower thermostability that could lead to a disintegration and expose cells to unchelated gadolinium. It is also conceivable that some of the contrast agent within the cells might slowly undergo a decomposition process, although our in vitro studies did not indicate any ill effects up to 2 weeks of culturing (Brekke et al., 2007a). Although GRID labelling for 8 h did not affect cell proliferation or viability in vitro, as a consequence of a delayed degradation of the chelate, it is possible that fewer cells survived in the long-term and reduced the efficacy of the transplant. Although previous studies that used relatively sub-acute imaging time points (up to 2 weeks following engraftment) in chronic lesions did not detect any ill effects on migration or differentiation (Modo et al., 2002a,b,c, 2004a,b), Brekke et al. (2007a,b) noted that a small fraction of GRID-labelled cells were engulfed by macrophages 2 weeks after implantation in a rat model of gliomas. Nevertheless, despite some of the cells dying, NSCs exerted a therapeutic effect. It is therefore possible that GRID-mediated toxicity leads to an

inflammatory response that will differentially affect gliomas and stroke damage. The increase in dead cells, especially close to the lesion cavity after a stroke, could have engendered a detrimental inflammatory response that could have lead to an increase in damage. We previously observed a 30% increase in lesion volume if dead stem cells were transplanted into an ischaemic lesion (Modo et al., 2003b). The development of imaging agents that can report on the functional status of cells (Himmelreich et al., 2006) might overcome these issues. Nevertheless, contrast agent-labelled cells were still present after 1 year in the injection tract and in the peri-infarct region. This indicates that at least in some cells, GRID did not decompose or have any deleterious effects. More stringent quality control measures and further in vitro studies will be needed to determine if the stability and/or purity of GRID could be increased to ensure a chronic survival of a greater number of transplanted cells or that a phased decomposition of the agent could be envisaged which might not interfere with cellular processes.

These results highlight the need for chronic imaging studies using contrast agents inside transplanted cells. Most contrast agents used to date for cellular imaging have not been specifically designed for this use and therefore might not be adequately suited for long-term tracking within the grafted cells. In vitro and short-term studies (<1 month) are inadequate to address these issues.

Neurological correlates of behavioural recovery

The clinical use of neural stem cells will mainly depend on the behavioural improvements that can be achieved. As previously reported, MHP36 cells recover (i.e. reduce) forepaw asymmetry in animals with stroke damage after contralateral implantation (Modo et al., 2002c; Veizovic et al., 2001). However, it remained unclear as to what areas in the brain are responsible for repair. A noteworthy difference between these two previous studies indicated that at 3 months following a stroke, transplanted cells did not reduce the lesion volume (Modo et al., 2002c), whereas by 1 year a significant 30% reduction in damage was observed (Veizovic et al., 2001). The current study using serial MRI and concomitant behavioural testing revealed that grafted cells exert a delayed effect on lesion volume. By 3 months following engraftment, there was no significant difference in lesion volume between MHP36-PKH26 grafted and MCAo-only animals, but a continued effect of transplanted cells resulted in a 35% reduction in lesion volume and an increase of 21% in ipsilateral striatal volume by 1 year.

Although grafted cells only affected lesion volume by 6 months, behavioural recovery on the bilateral asymmetry test preceded the reduction in lesion volume and was complete by 12 weeks following implantation. Recovery of the somatosensory impairment therefore must be subserved by a different neural substrate. A multiple regression analysis identifying changes in anatomical structure that predict performance on the bilateral asymmetry test indicated that forepaw bias is due to a combination of the extent of damage and the size of ipsilateral striatum, whereas the total amount of time it took animals to remove both sticky tapes is a function of the size of the evolution of striatal asymmetry and changes in the contralateral striatum. We have previously identified the contralateral striatum as a site that is undergoing an upregulation of apolipoprotein E after NSC transplantation possibly by

inducing plasticity in this area (Modo et al., 2003a). Change in the cortex was not associated with forepaw asymmetry, although we previously reported a strong correlation between degree of cortical damage and performance on the bilateral asymmetry test in a purely cortical lesion (Ashioti et al., 2007). It is likely here that the degree of damage to the ipsilateral striatum is more important than cortical damage. Although processing of somatosensory information is localised to the somatosensory cortex, information needs to pass through the somatosensory and motor loop of the striatum (Alexander et al., 1986). If this loop or 'relay station' is impaired, the somatosensory system cannot function effectively. Therefore, it is a combination of a lesion (this includes cortical damage) and striatal volume that predicts performance on the bilateral asymmetry test rather than cortical damage or sparing. Transplanted cells integrate in both the cortex and striatum whilst differentiating into appropriate phenotypes (Modo et al., 2002c) and are already present in the peri-infarct area by 1–2 weeks following engraftment (Modo et al., 2004a). It is therefore likely that it is this engraftment of sufficient cells that drives the somatosensory recovery evident by 4–6 weeks post-grafting.

Caution for clinical implementations

Being able to identify the neural substrates of recovery will provide the basis for optimising implantation whilst also allowing a serial non-invasive monitoring of therapy in patients. To ensure that cells indeed repair, it is desirable to track their movement and survival in patients. Cellular MRI can provide this information non-invasively over protracted time points. Although extensive preclinical studies have been conducted, little information is available regarding the long-term effects of contrast agents inside of cells and their potential implications on functional recovery. Although there is less concern regarding the use of contrast agents in cells that only need to be present temporarily to exert their therapeutic effect, such as dendritic cells in melanomas (de Vries et al., 2005), the use of neural cells demands a greater degree of caution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Animals with stroke damage show a very significant asymmetry in the removal of adhesive tape from the forepaws compared to controls. Upon transplantation of neural stem cells from the MHP36 cell line, only animals with MHP36 cells labelled with PKH26 showed a significant recovery compared to stroke only animals. Labelling of MHP36 cells with GRID did not result in the same recovery. Both stroke only and the MHP36+GRID groups spontaneously improved their forepaw asymmetry by 26 weeks and no longer exhibited a deficit compared to controls or MHP36+PKH26 transplanted animals. In contrast, the total time of adhesive removal did not exhibit an initial difference between stroke only and control animals. Only 26 weeks following transplantation, when the asymmetry bias disappeared did stroke only animals reveal a significant difference to control and MHP36+PKH26-transplanted animals. MHP36+PKH26 animals showed the same total removal times. MHP36+GRID-transplanted animals performed at an intermediate level between controls and stroke only animals. This suggests that in MHP36+GRID animals, MHP36 cells might have exerted early beneficial effects that might be reflected in later behavioural performance.

Figure 2.

(A) shows the evolution of T₂-weighted MR images of animals within the different experimental groups over 52 weeks post-transplantations. Progressively over the 6 weeks post-ischaemia (4 weeks post-grafting), the lesion environment develops into a lesion cavity that is clearly defined on T₂-weighted scans. However, little change in the MRI signature and size of the lesion can be observed beyond this time. (B) Already 1 week post-transplantation, GRID-labelled cells can be detected on T₂-weighted MR images surrounding the vicinity of the lesion (left-facing arrows). Labelled-cells did not constitute

the border to the cavity, but were located within intact-appearing tissue. One month following implantation, these GRID-labelled cells were located at the edge of the lesion indicating that GRID-labelled cells did not integrate into tissue undergoing subsequent degeneration. It is likely that this tissue was already severely gliotic and that this inhibits infiltration of stem cells. In 37% of cases, edema (as indicated by a hyperintensity) can be seen at the site of injection (left-facing arrows) and this prevents detection of transplanted cells labelled with GRID which is apparent 1 month following injection when the edema receded. PKH26-labelled cells were not visible on these scans. (C) GRID-labelled cells are detectable by MRI for up to 1 month following injection, but can no longer be detected on images 3 months post-transplantation. The migration pattern that can be observed on these scans indicates that depending on the extent and location of the lesion, transplanted cells either remain at the outer border of the lesion or in case of a subcortical infarct, cells migrate around the cavity and will surround the cavity. It is interesting to note that migration to the lesion always seems to be an extension of the corpus callosum that these cells use to migrate. Grafted cells do not appear to shortcut through the striatum to the site of the lesion indicating a key role of the corpus callosum in cell migration to the site of infarction.

Figure 3.

Evolution of anatomical structures. To determine how transplanted cells affect the evolution of brain growth, lesion, striatum and cortex, total volumes prior to transplantation were used as baseline to assess percentage change over time. This removes the intra-animal variability and provides a more powerful assessment of treatment effects. Most significantly here, PKH26-labelled MHP36 cells reduced the size of the lesion by 35% 1 year post-grafting, whereas cells labelled with GRID did not reduce lesion size. Similar effects were seen in the

striatum and cortex. Interestingly, no dramatic effects were observed on the asymmetry between ipsi- and contralateral striatum or cortex.

Figure 4.

Ex vivo MRI. To provide high-resolution 3 dimensional images of the GRID-transplanted brains, ex vivo MR images were acquired. (A) Even after 1 year the injection tract can still be clearly seen in the damaged brain although in vivo this area was no longer detectable after 3 month. Ex vivo images are less susceptible to intravoxel movement, have a higher resolution to avoid partial volume effects and additional averages can be included to improve detection of smaller clusters of cells compared to in vivo limitations. (B) In the lesion cavity, filamentous elements can be observed on ex vivo images that were not evident on in vivo images. It is possible that these reflect parts of the glial scarring, but their location and distribution strongly suggest that these might be remnants of blood vessels that were reperfused and viable although the surrounding neuropil died away.

Figure 5.

Histological assessment of stroke damage and stem cell implantation. (A) Transplanted cells as identified by GRID's red fluorescence can still be seen in the injection tract and the horn of the lateral ventricle (lv) with some of the MHP36 still in corpus callosum. (B) GRID-labelled cells could also still be found in the peri-infarct area. However, far fewer cells could be found here than in previous experiments (Modo et al., 2004a). (C) In the cortex, however, red autofluorescence was also present in normal control animals. (D) This punctate red inclusions had the same appearance than GRID or PKH26 and did not allow a reliable

identification of transplanted cells in these areas. (E) Sudan Black staining can remove this autofluorescence by tainting the inclusions black as can be seen here on the brightfield image. However, Sudan Black also covers the fluorescent properties of GRID and PKH26 and therefore still precludes identification of grafted cells.