Effects of Implantation Site of Stem Cell Grafts on Behavioral Recovery From Stroke Damage

Michel Modo, PhD; R. Paul Stroemer, PhD; Ellen Tang, BSc; Sara Patel, PhD; Helen Hodges, PhD

Background and Purpose—Findings that MHP36 stem cells grafted into intact parenchyma contralateral to the lesion induced by middle cerebral artery occlusion promoted recovery from stroke deficits led us to investigate whether implantation site of stem cells affects the functional efficacy of MHP36 grafts.

Methods—MHP36 cells (200,000/μL) were implanted in the left (n=8) or right (n=9) parenchyma or infused into the right ventricle (intraventricular; n=7) 2 to 3 weeks after stroke induced by 60 minutes of intraluminal right middle cerebral artery occlusion. Additionally, intact (n=11) and stroke (n=7) control groups were tested for 14 weeks in bilateral asymmetry, rotation bias, and spatial learning tasks before histological investigation of cell distribution and differentiation.

Results—Rats with left and right parenchymal grafts showed reduced bilateral asymmetry but no improvement in spatial learning. Conversely, spatial learning improved in rats with intraventricular grafts, but marked asymmetry persisted. No grafted group showed reduced amphetamine-induced rotation bias or reduced lesion volume relative to stroke controls. In all grafted groups, cells occupied both sides of the brain. A third of cells grafted in the striatum crossed the midline to occupy homologous regions in intact and lesioned hemispheres and differentiated into site-appropriate phenotypes.

Conclusions—After stroke, both the intact and lesioned hemispheres attract grafted stem cells, suggesting repair processes that utilize cells both for local repair and to augment plastic changes in contralateral motor pathways. However, differential effects of parenchymal and intraventricular grafts suggest that different mechanisms are implicated in recovery from cognitive and sensorimotor deficits induced by stroke. (Stroke. 2002;33:2270-2278.)

Key Words: brain tissue transplantation □ cell differentiation □ middle cerebral artery occlusion □ recovery of function □ stem cells

Cell replacement therapy offers a novel potential treatment for stroke damage.1 Both fetal2 and neuroteratocarcinoma grafts3 improve functional deficits after experimental stroke in the rat. With their capacity to colonize regions of brain damage and to differentiate into appropriate phenotypes under host guidance, conditionally immortalized neuroepithelial stem cells are promising candidates for repair of stroke damage.4 Veizovic at al.5 have shown long-term recovery from deficits induced by 60-minute occlusion of the middle cerebral artery (MCA) after grafts of the transgenic murine stem cell line MHP36 were placed in the intact hemisphere contralateral to the stroke lesion to avoid exposure to the inflammatory environment of the developing lesion. Cells did not remain at the site of implantation but migrated widely through the striatum and somatosensory cortex in both the intact and lesioned hemispheres, with approximately a third of cells crossing from the intact to the lesioned side. The presence of cells on both sides of the brain suggested that functional recovery might involve both limited repair of pathways on the damaged side and interactions with processes of reorganization in the intact side.

It is therefore necessary to compare the functional effects and patterns of migration after grafts on both sides to clarify these mechanisms. If cells are chiefly attracted by signals from direct brain damage, then ipsilateral grafts would remain in the lesioned hemisphere, while a proportion of cells grafted contralaterally would migrate across the midline, as observed by Veizovic et al.3 If cells respond to signals from brain undergoing reorganization contralateral to stroke damage, as imaging studies have demonstrated in stroke patients,6 then some cells from ipsilateral grafts would be expected to migrate to the intact hemisphere. Furthermore, it is relevant to compare the functional effects and distribution of cells infused into the ventricles with those grafted into the parenchyma, since intraventricular grafts would provide a less invasive surgical procedure and might enable grafted stem cells to integrate into a migratory pathway utilized by indigenous stem cells after neurogenesis in immature and adult brains.7

The present study investigated the effects of implantation site (ipsilateral, contralateral, or intraventricular) with the use of the clonal MHP36 cell line, derived from the H2K₄-tsA58
transgenic mouse neuroepithelium,8 in rats subjected to 60-minute occlusion of the right MCA. Tasks were chosen to reflect stable impairments in sensorimotor, motor, and cognitive function.5 After behavioral testing was completed, brains were analyzed for lesion magnitude, distribution of transplanted cells, and their differentiation into neuronal phenotypes.

Materials and Methods

Animals
Forty-two Sprague-Dawley rats (Charles River, UK), (weight, 270 to 330 g at surgery) were maintained on a 12-hour light/dark schedule (lights on at 8 AM), with food available ad libitum until recovery from surgery and then restricted (10 g/100 g) to standardize weight. Procedures complied with the UK Animals (Scientific) Procedures Act (1986) and the Ethical Review Process of the Institute of Psychiatry.

MCA Occlusion and Transplant Surgery
The right MCA was occluded for 60 minutes by insertion of a round-tipped polypropylene (Prolene) filament via the common carotid artery to the ostium of the MCA in the circle of Willis. Halothane (in 70% N2O/30% O2) anesthesia was used for insertion and removal of the filament, with temperature held at 37±1°C by rectal probe and heating pad. During occlusion rats were assessed for forelimb flexion and contralateral circling to confirm ischemia. During the recovery period, rats were tested for neurological deficits and lesion asymmetry baselines.9

Rats were assigned to groups showing equivalent forelimb flexion9 to receive cells grafted into the lesioned (ipsilateral; n=8) or intact (contralateral; n=9) striatum or the right ventricle (intraventricular graft; n=7) to 2 weeks after MCA occlusion (MCAO). Sham-occluded (n=11) and stroke (n=7) animals served as controls.

MHPC66 cells from frozen stock (passage 42) were prelabeled by incubation for 4 minutes with the fluorescent cell membrane marker PKH26 (Sigma)10 and suspended (25 000 cells per microliter) with 1 mmol/L N-acetyl-l-cysteine (Sigma) in Hanks’ balanced salt solution (Gibco). Cell viability, determined by trypan blue (Sigma), was calculated as the total number of anticlockwise turns divided by the number of clockwise turns in both directions.

Behavioral Assessment
The test battery was identical to that used by Veizovic et al.5 Time courses for procedures are provided in Table 1.

The bilateral asymmetry test of sensorimotor dysfunction measured the disparity in time taken to remove sticky tape strips (0.8×6 cm) from the affected and unaffected forepaws for 180 seconds.9

The rotameter (TSE GmbH) measured motor rotation asymmetry in response to amphetamine (2.5 mg/kg) and apomorphine (1.0 mg/kg) 30 minutes before testing, which stimulate presynaptic and postsynaptic dopamine receptors, respectively, on the intact left side to induce marked rightward circling in stroke animals. Rotation bias was calculated as the total number of anticlockwise turns divided by the total number of clockwise turns, and total activity was calculated by summing turns in both directions.

Statistical Analyses
Results for the water maze and bilateral asymmetry tests were analyzed by repeated-measures ANOVA (SPSS), with groups as the between-subjects factor and trials/days as the within-subjects factor. We used t tests to analyze postlesion bilateral asymmetry deficits. One-way ANOVAs were used for group differences on the rotameter, the water maze probe trial, lesion volume, and differences in cell counts in ROI. The Bonferroni post hoc test was used to compare groups.

Learning to find the safe platform in the water maze (4 trials of 60 seconds each per block of testing, 200-cm-diameter pool, 10-cm-diameter platform, water at 24±2°C) provided a measure of spatial navigation deficits (in time taken to find the platform, distance, and swimming speed) as recorded by an image analysis system (HVS Image Ltd).5

Histology
Animals were overdosed with pentobarbitone sodium (Animal Care Ltd), flushed transcardially with heparinized saline, and perfused with 4% paraformaldehyde in 0.2 mol/L PBS. Brains were cryoprotected by 30% sucrose and cut by cryostat into 50-μm sections, a thickness chosen to avoid distortion or breakage that might occur with thinner sections of cavitated brain.

Lesion volume was calculated in every 20th section by Simpson’s rule with the use of digital microscope images analyzed by Image Pro Plus (Media Cybernetics) and expressed as a percentage of total brain volume. Transplanted cells were identified by the fluorescent PKH26 label under confocal microscopy (Leica). To determine graft survival semiquantitatively, transplanted cells in the same focal plane were counted (×400 magnification) bilaterally in 3 regions of interest (ROIs): somatosensory cortex, striatum, and septum at the level of implantation (+0.7 mm from bregma) in a rectangular field (0.202×0.252 mm) selected to provide an appreciation of graft survival within areas equivalent relative to implantation tracts in each ROI for each brain (Figure 5A). Since it is difficult to measure thinly scattered grafted cells, the purpose of standardized ROI cell counts was not to estimate the total number of grafted cells but to obtain an accurate count within selected ROIs across groups.

Differentiation of transplanted cells was determined by colocalizing phenotypic markers for different cell types (Table 2) with PKH26 as a marker for transplanted cells. Primary antibodies were incubated overnight at room temperature before either the fluorescent anti-mouse or anti-rabbit Alexa 488 (Molecular Probes; 1:500) secondary antibody was applied for 1 hour. To determine neuronal differentiation, cells colocalizing with PKH26 were counted after counterstaining with PKH26-positive cells within the same ROI.

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TABLE 1. Timetable of Experiment

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Procedure</th>
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<tbody>
<tr>
<td>1</td>
<td>MCAO surgery</td>
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<tr>
<td>2–3</td>
<td>Bilateral asymmetry test: lesion baseline</td>
</tr>
<tr>
<td>3–4</td>
<td>Transplant surgery</td>
</tr>
<tr>
<td>5–15</td>
<td>Bilateral asymmetry test: graft effects</td>
</tr>
<tr>
<td>1–10</td>
<td>2 trials (180 s each) per week</td>
</tr>
<tr>
<td>12–15</td>
<td>Rotameter</td>
</tr>
<tr>
<td>15–17</td>
<td>Water maze</td>
</tr>
<tr>
<td>18</td>
<td>Perfusion</td>
</tr>
<tr>
<td>&gt;19</td>
<td>Histological assessment</td>
</tr>
</tbody>
</table>

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Results

Behavioral Assessment

Bilateral Asymmetry Test

After stroke surgery, animals removed the left tape significantly more slowly than the right tape ($t=4.162$, $df=64$, $P<0.001$), whereas there was no difference between removal times for controls. After grafting, the difference between paws remained substantial in stroke controls and in rats with intraventricular grafts but gradually decreased in rats with parenchymal grafts. Over the last 4 weeks, rats with parenchymal grafts no longer differed in speed of swimming ($F_{4,5}=24.267$, $P<0.001$) but not grafted striatal groups, indicating an intermediate speed.

Rotameter

There was no difference between groups in baseline (saline) rotation bias (Figure 2), although a modest overall effect of group ($F_{4,38}=2.722$, $P<0.05$) suggested a tendency to bias in stroke controls. In contrast, injection of amphetamine ($F_{4,37}=5.283$, $P<0.01$) and apomorphine ($F_{4,38}=6.378$, $P<0.001$) induced a significant bias in all stroke groups compared with controls ($P<0.01$), which was not corrected in any grafted group. There were no differences in total activity between groups under saline ($F_{4,38}=1.199$, $P=NS$), amphetamine ($F_{4,38}=1.888$, $P=NS$), or apomorphine ($F_{4,38}=1.768$, $P=NS$) treatment, indicating that locomotor activity did not significantly differ between groups.

Water Maze

Controls found the platform significantly faster ($F_{4,4}=23.819$, $P<0.001$) than rats with ipsilateral grafts ($P<0.001$), rats with contralateral grafts ($P<0.001$), or stroke controls ($P<0.001$). In contrast, rats with intraventricular grafts found the platform as rapidly as controls (Figure 3) and were significantly superior to rats with contralateral grafts ($P<0.001$), rats with ipsilateral grafts ($P<0.001$), and MCAO controls ($P<0.001$). Rats with intraparenchymal grafts and stroke controls showed comparable impairments (Figure 3). Similar results were seen for path length ($F_{4,4}=24.267$, $P<0.001$), although rats with intraventricular grafts were marginally impaired ($P<0.05$) relative to controls while superior to all other stroke groups ($P<0.01$). Groups differed in speed of swimming ($F_{4,4}=13.504$, $P<0.001$), since controls swam more slowly than all other groups ($P<0.01$, except for the intraventricular group, in which $P<0.05$), suggesting that accurate searching reduced speed. Rats with intraventricular grafts swam more slowly than stroke controls ($P<0.05$) but not grafted striatal groups, indicating an intermediate speed.

Histology

Lesion Volume

All groups with MCAO showed substantial damage, averaging 84 mm$^3$ (18% of total brain volume), while control

TABLE 3. Statistical Summary of Paw Differences in the Bilateral Asymmetry Test

<table>
<thead>
<tr>
<th>Groups</th>
<th>Repeated-Measures ANOVA</th>
<th>Post Hoc t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start (Week 1–4)</td>
<td>End (Week 7–10)</td>
</tr>
<tr>
<td></td>
<td>$F$</td>
<td>$df$</td>
</tr>
<tr>
<td>Ipsilateral</td>
<td>11.586</td>
<td>1; 4</td>
</tr>
<tr>
<td>Contralateral</td>
<td>17.033</td>
<td>1; 4</td>
</tr>
<tr>
<td>Ventricular</td>
<td>29.634</td>
<td>1; 4</td>
</tr>
<tr>
<td>MCAO only</td>
<td>35.785</td>
<td>1; 4</td>
</tr>
<tr>
<td>Controls</td>
<td>1.806</td>
<td>1; 4</td>
</tr>
</tbody>
</table>

GFAP indicates glial fibrillary acidic protein; ChAT, choline acetyl transferase.
surgery resulted in 2% tissue loss. Thus, controls differed ($P<0.001$) from all stroke groups ($F_{4,34}=9.775$, $P<0.001$). However, there were no differences between stroke groups, indicating that transplants had not modified lesion volume at 12 weeks after grafting. Lesions typically extended from +2.2 to −5.5 bregma and included the ipsilateral motor and sensorimotor cortices and mediodorsal striatum. Secondary degeneration was indicated by thalamic atrophy, but no damage to the hippocampus was apparent.

**Distribution of Transplanted Cells**

In all regions of interest, grafted cells differentiated into both neurons and astrocytes (Figure 4). Figure 5A shows the distribution of grafted cells mapped on a template reflecting typical 60-minute MCAO damage. Pooled data from all ROIs ($F_{2,98}=7.818$, $P<0.001$) showed that a greater number of cells survived in ROIs after contralateral than ipsilateral ($P<0.001$) or intraventricular ($P<0.05$) implantation; ipsilateral and intraventricular grafts did not differ in cell counts within these sampled regions (Figure 5B). Greatest cell numbers were seen in the contralateral striatum ($F_{2,18}=14.762$, $P<0.001$), which harbored more cells from contralateral than from ipsilateral ($P<0.01$) or intraventricular ($P<0.05$) grafts. In both groups of rats with parenchymal grafts, approximately a third of cells migrated to the other side of the brain, while two thirds remained within the grafted side. Hence, bilateral comparisons of ROI differed in rats with ipsilateral ($F_{4,4}=5.04$, $P<0.01$) and contralateral ($F_{4,4}=4.163$, $P<0.01$) grafts. However, ROI did not differ in rats with intraventricular grafts ($F_{4,4}=1.769$, $P=NS$) because cells spread evenly from the midline infusion site.

**Neuronal Differentiation of Transplanted Cells**

There was no effect of transplant site on the overall percentage of grafted cells that differentiated into neurons (30% PKH26/NeuN positive with all 3 sites of grafting) in the sampled regions, percentages within each ROI (Figure 5C), or absolute number of neurons in each ROI (Figure 5E). However, neuronal phenotype was differentially expressed in ROIs ($F_{4,9}=4.390$, $P<0.01$) because fewer grafted cells developed into neurons in the ipsilateral and contralateral somatosensory cortex than in medial structures (septum and striatum; $P<0.05$).

**Phenotypes of Grafted Cells**

Transplanted cells differentiated into site-appropriate phenotypes. In the striatum, transplanted cells differentiated to a large degree into interneurons (Figure 6A) and also into GABAergic output neurons that are DARPP-32 positive (Figure 6B). Grafted cells were also surrounded by fibers innervating the striatum, such as tyrosine hydroxylase–positive dopaminergic fibers. Nesting of PKH26-positive cells within the matrix of input fibers suggests that innervating host neurons formed synaptic connections with grafted cells. The compartmentalized nature of the striatum helped to determine whether grafted cells expressed uncharacteristic phenotypes (as determined by the markers used in this study). No atypical phenotypes were seen, and differentiation of grafted cells always paralleled the host phenotypes within a particular region of the striatum.

**Discussion**

These results suggest that the type of functional recovery after MCAO depends on the site of implantation and on the demands of the task. No recovery of sensorimotor function was seen in rats with intraventricular grafts, whereas animals with intraparenchymal grafts either ipsilateral or contralateral to the lesion showed reduced sensorimotor asymmetry. Conversely, rats with intraventricular grafts learned to locate the submerged platform in the water maze as efficiently as...
controls, whereas rats with intraparenchymal grafts failed to show this cognitive recovery and performed like stroke controls. Stroke controls showed a pronounced rotation bias in response to amphetamine, but this was not normalized by grafts. This pattern of results, particularly the dissociation between intraparenchymal and intraventricular grafts, suggests that several mechanisms may be involved in recovery from the effects of stroke damage.

Histological examination revealed 4 key findings: (1) cells migrated to the intact and lesioned side of the brain regardless of implantation site; (2) more cells survived with contralateral than ipsilateral or intraventricular grafts; (3) approximately 30% of cells differentiated into neurons in the selected ROIs and exhibited all the major phenotypes of the normal striatum; and (4) lesion volume was not reduced in grafted rats. These findings carry several implications for the mechanisms of graft effects.

**Cell Distribution and Number**
A large proportion of intraparenchymal grafted cells (30% to 35%) migrated across the midline not only from the intact to the lesioned hemisphere, as found by Veizovic et al, but also from the lesioned to the intact side, contrary to our previous belief that stem cells only migrate to sites of injury. This novel finding indicates that denervation and reorganization in the so-called intact hemisphere generate signals that are as attractive to stem cells as ischemic injury. The finding that cell counts within ROIs were significantly greater with contralateral implantation supported our view that grafting into the intact striatum might foster cell survival. However, ipsilateral grafts were as effective as contralateral grafts in reducing bilateral asymmetry, so that increased cell numbers did not translate into greater functional recovery, possibly because cells in ipsilateral striatum differentiated more readily into neurons. Cell number per se also cannot account for differences in effects of parenchymal versus intraventricular grafts, since numbers in intraventricular grafts were similar to those in ipsilateral grafts. It should be noted, however, that the present study only used semiquantitative measures that could underestimate or overestimate graft survival or differentiation. The extensive migration and the extent of structures affected by MCAO damage render a quantitative or stereological approach technically difficult, and use of 2-dimensional analyses as a method of providing...
reasonable “snapshot” representations of cell distribution has been advocated.\textsuperscript{11}

The reduction in bilateral asymmetry possibly depended on sufficient engraftment of the striatum that was provided by parenchymal grafts on either side. This possibility is supported by our findings\textsuperscript{12} that apolipoprotein E, a lipid transporter associated with both clearance of dead cells and neuronal reorganization after stroke,\textsuperscript{13} was upregulated in the contralateral striatum of rats with parenchymal but not intraventricular grafts and seen in both host and grafted neurons and astrocytes. These results are consistent with our previous suggestion that correction of motor asymmetry involves interactions with striatal regions undergoing both degeneration and reorganization after stroke, to foster repair of local pathways and to stimulate host reorganization of normally silent contralateral corticospinal links. Conversely, concentration of intraventricular grafts in the septum, an area that governs cholinergic and GABAergic projections to the hippocampus, may have improved communications with this important structure for spatial learning, whose projections are disrupted by MCAO, even though gross damage may not be apparent.

**Cell Differentiation and Phenotype**

Findings that approximately 30\% of grafted cells differentiated into neurons in ROIs after ipsilateral, contralateral, or intraventricular grafting indicate that the overall proportion of neurons is not a correlate of differential graft efficacy. However, attempts to determine whether specific phenotypes of grafted cells relate to their functional effects were also not successful in discriminating between grafts, since similar phenotypes were seen in all groups. Transplanted MHP36 cells differentiated to a large extent into \textsuperscript{2+}-binding protein–positive interneurons and GABAergic output neurons, reflecting the general proportion of these cells in the striatum. Double labeling clearly identified grafted cell types at high magnification, with confocal
slices and rotation to clarify markers within cells (Figure 6). However, with thinly scattered cells, confocal fluorescence may not be appropriate to quantify cell types at this level of detail throughout ROIs. Thus, although double-labeled cells were identified, accurate comparisons of numbers were not feasible, and it remains to be seen whether implantation site differentially influenced mature phenotype expression.

**Lesion Volume**

Veizovic et al reported that contralateral MHP36 grafts significantly reduced infarct volume by 36% at 11 months after grafting. However, the present results showed no evidence for a significant reduction in lesion volume in any of the grafted groups at 3 months after grafting, a time at which lesion volume itself was smaller than at 11 months (18% as opposed to 26% of total brain volume).
Taken together, these results suggest that the effects of MHP36 grafts on stroke volume do not involve an early cerebroprotective mechanism to prevent the development of ischemic cell death but might limit the progression of cavitation and secondary damage distant from the infarct in the long run. Veizovic et al also found that dopamine agonist-induced rotation bias was normalized in rats with contralateral grafts at 38 to 45 weeks after grafting, whereas no reduction in bias was seen at 4 to 7 weeks in the present study. Thus, reduced rotation may be a late effect and, if so, may be related to delayed graft effects on lesion volume. The bias on the bilateral asymmetry test also appeared to include a motor deficit, whereas the present study only detected a sensorimotor bias. In stroke animals the initial lack of response to the affected paw induces a serial strategy to remove the adhesive tapes, whereas controls have to resolve response competition between paws. Motor impairments indicated by increases in removal times were not apparent in the present studies, but the results clearly show a difference between paws indicative of a sensorimotor bias that was reduced after intraparenchymal grafting but not intraventricular grafting. A time course study is therefore needed to evaluate the contribution of the evolution of stroke damage to effects of grafted cells on lesion volume and behavioral outcome, in interaction with site of transplantation.

Conclusions
This study has shown that within a 3-month period, intraparenchymal stem cell grafts improved sensorimotor dysfunction, whereas intraventricular grafts promoted normal spatial learning in rats after MCAO, indicating that graft placement critically affects the type of functional recovery that occurs. Histological measures such as grafted cell number, distribution, neuronal differentiation, and lesion volumes were not obviously related to differences in graft effects. However, a substantial proportion of grafted cells migrated to the opposite side of the brain, whether this was lesioned or intact, indicating that stem cells are attracted by and interact with regions of both degeneration and reorganization. Differential density of grafted cells in the striatum and septum with intraparenchymal and intraventricular grafts may offer clues to their different functional effects, which should be confirmed by more detailed quantification of possible differences in phenotype. While these results raise many questions, they indicate that several sites of implantation may be optimal for

Figure 6. Confocal images of neuronal phenotypes of transplanted cells in the striatum. Transplanted cells (PKH26 in red) appropriately differentiated into interneuronal phenotypes expressing choline acetyl transferase (ChAT; A to C), and the Ca\(^{2+}\)-binding proteins parvalbumin (Parv; D to F), somatostatin (Som; G to I), and calretinin (Cal; J to L). Transplanted cells also differentiated into striatal GABAergic output neurons (DARPP-32; M to O) and were found to be enclosed by markers for substance P (Sub P; V to X), tyrosine hydroxylase (TH; P to R), and enkephalin (Enk; S to U), indicating that transplanted cells differentiated into site-appropriate phenotypes and were integrated into the host neuropil.
different impairments and that several mechanisms may be involved in stem cell repair of widespread stroke damage.

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References
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