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Hematopoietic Stem Cell Transplantation Protects Mice From Lethal Stroke

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Abstract

Stroke is a major cause of mortality and morbidity in the United States. The ideal therapeutic approach would minimize cell death and regenerate brain tissue. In order to investigate some questions that are related to such an approach, we have generated a mouse model in which we induce a stroke using the Middle Cerebral Artery Occlusion method. After 2h occlusion followed by reperfusion, 99% of mice died within 8 days of stroke. Total bone marrow cell transplantation by intravenous injection revealed an optimal timing of cell transfer in two doses on days 1 (same day of surgery) and 2 after surgery. Moreover, intravenous injection of Sca1+ bone marrow cells (enriched in hematopoietic stem cells) showed a dose-response effect on survival. Surviving mice also had no signs of apparent paralysis or weakness. Tracking analysis using donor stem cells expressing LacZ revealed only few donor cells in the brain. We conclude that hematopoietic stem cell-rich Sca1+ bone marrow cell transplantation after stroke protects the brain of a sizeable portion of mice subjected to stroke and alleviate remarkably the resulting neurological morbidity in surviving mice.

Keywords

Stroke; hematopoietic stem cells; transplantation; neuroprotection; survival

Introduction

Stroke is one of the most common causes of death and severe disabilities in developed countries (Davenport and Dennis, 2000). Stem cell therapy has been used with variable outcomes both in clinics (Bang et al., 2005; Savitz et al., 2004) and in experimental stroke (mice and rats) (Chen et al., 2003; Borlongan et al, 2004; Gage et al, 1995; Liu et al, 2006; Honna et al, 2006; Zin'Kova et al, 2007; Wei et al, 2007; Ukai et al, 2007; Yanagisawa et al, 2006; Hayashi et al, 2006; Tagushi et al, 2004; Nystedt et al, 2006; Willing et al, 2003; Jiang et al, 2006; Comi et al, 2008; Lee et al, 2007; Kelly et al, 2004; Guzman et al, 2008). Interestingly, the

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infarct size impact in the brain was quite variable between studies, ranging from <50% of the hemisphere (Hanna et al, 2006; Nystedt et al, 2006) to > 50% of the hemisphere (Ukai et al, 2003; Jiang et al, 2006), often decreasing after stem cell transplantation (Hanna et al, 2006; Ukai et al, 2003; Jiang et al, 2006), but sometimes remaining unchanged (Nystedt et al, 2006). The difference in the outcome could be related to the initial lesion, the modality of transplantation (intravenous vs intracranial injection) or type of transplanted cells (human CD34+ cells, murine hematopoietic, mesenchymal, neural, or embryonic stem cells). Indeed, some studies used neural stem cells (NSCs) from rodents (Jiang et al, 2006; Comi et al, 2008; Guzman et al, 2008; Gage et al, 1995) or human (Kelly et al, 2004; Lee et al, 2007), or primate origin (Hayashi et al, 2006), mesenchymal stem cells (MSC) from mouse (Veng et al, 2008; Zin'kova et al, 2007; Wei et al, 2007; Ukai et al, 2007) and human (Liu et al, 2006; Honna et al, 2006), mouse ES cells (Yanagisawa et al, 2006), and human hematopoietic stem cells (HSCs) (Nystedt et al, 2006; Willing et al, 2003; Tagushi et al, 2004). While the primary focus in previous studies ranged from elucidating the fate of donor cells and infarct size to the description of functional recovery, to our knowledge, the ability of transplanted stem cells to rescue mice from a lethal injury has never been reported. Furthermore, there has been no study evaluating murine HSCs in such a rescue experiment.

In this study, using the MCAO technique, we generated a mouse model for stroke with a large reproducible infarct size that resulted in the death of almost all mice within 8 days of surgery, and demonstrated that the intravenously (IV) administered murine Sca1+ stem and progenitor cells can rescue ~40% of animals and reduce infarct size by $\geq 50\%$. Our study provides the first evidence that murine HSC-rich Sca1+ bone marrow cell population plays a major role in protecting mice from death after induction by a stroke model. We also showed that the timing of the transplantation is important for the success of the therapy.

Materials and methods

Mouse strains

Mice used were obtained from the Jackson laboratories (Jackson Laboratories, Bar Harbor, ME, USA). Animals used for generating the stroke model were male C57BL/6J, 8–10 weeks of age. Mice used as stem cell donor were either male C57BL/6J, or male ROSA26 mice on a C57BL/6J background, 8–12 weeks of age. All mice were housed in a pathogen-free environment, and procedures were conducted in compliance with the Guidelines of the Institutional Animal Care and Use Committee (IACUC).

Generating the stroke model

The stroke model was generated using the middle cerebral artery occlusion (MCAO) method, as previously described (Hess et al, 2002). Briefly, mice were anesthetized and the right carotid arteries exposed. The common carotid artery (CCA) and external carotid artery (ECA) were ligated, and a small incision done in the CCA through which a silicon rubber-coated monofilament 6-0 was inserted (diameter with coating $0.31 \pm 0.02\text{mm}$, length of coating 5–6 mm, Docol Corporation) and pushed 11 mm inside of the internal carotid artery (ICA) to block the middle cerebral artery (MCA). The CCA and ICA were ligated to secure the filament in place and prevent bleeding. In permanent MCAO, the filament was kept in place, while in transient MCAO, it was withdrawn after variable occlusion time (2h, 4h) to re-establish blood flow through the MCA. The CCA was ligated afterwards to prevent bleeding. Mice were sutured and observed afterwards. Animal temperature was monitored throughout the procedure.

Detecting and quantifying the infarct

Mice were sacrificed by halothane inhalation and decapitated. Whole brains or 1 mm slices were stained with 2% 2,3,5, triphenyl tetrazolium chloride (TTC) for 30 min at 37°C, and then fixed with 4% paraformaldehyde, as previously described (Bederson et al, 1986; Yanamoto et al, 2003). Live tissue stains red while infarct areas appear white. The infarct volume was quantified using ImageJ software, and expressed as a percentage relative to the ipsilateral hemisphere.

Assessing the mouse functional status

Neurological impairment was assessed 2h after recovering from anesthesia, then 7 days and 14 days later in surviving animals. The scoring system was used as previously described (McCullough et al, 2005) with slight modifications: for each sign, the grade “0” was given if the sign is not detected, and “1, 2, 3, or 4” given respectively to each of the following signs: torso turned towards the right, circular motion towards the right, mouse can’t bare weight i.e. leaning down at the side of paralysis, barrel rolling precipitated by slightly lifting the mouse by the tail or placing it on its back. The final score was the sum of the grades of all 4 signs, ranging from 0 (normal) to 10 (highly impaired). In addition to this score, the righting reflex was tested by placing the mouse on its back: when the mouse displays a random motion before righting up it is given the sign “+” i.e. impaired; if it rights up normally it is given the sign “-” i.e. not impaired. Mice not showing neurological deficit (circular motion) 2h after recovery from anesthesia were excluded from the study.

Hematopoietic stem cell transplantation (HSCT)

At various times after the surgery (2h, 1 day, 2 days, 3 days, 4 days) MCAO mice were subjected to intravenous injection of either total bone marrow cells or *Sca1*⁺-enriched bone marrow cells without prior immunosuppression. The *Sca1*⁺-enriched cell population includes hematopoietic stem and progenitor cells, excluding most differentiated cells and inflammatory cells (Wilson et al, 2007). Briefly, bone marrow was extracted from long bones of healthy donor mice (C57BL/6J or ROSA26 on C57BL/6J background, 8–12 weeks of age) and red blood cells were lysed with RBC lysis buffer (Sigma). Cells were then either directly used for bone marrow transplantation (BMT), or processed for the positive selection of *Sca1*⁺ cells using the EasySep Mouse *Sca1* Selection Cocktail (Stem Cell Technologies, Vancouver, Canada), whereby the cells are labeled with anti-*Sca1* antibody that is coupled to the fluorescent dye Phyco-Erythrin (PE). Samples of the cells before and after enrichment were passed through a flow cytometer to assess the enrichment using Phyco-Erythrin labeling, which showed ~6–7-fold enrichment of the stem and progenitor cells (Figure 2). Total bone marrow cells or *Sca1*⁺ cells were injected intravenously in 50–100 µl PBS in a lateral tail vein.

Tracing of donor cells

Sca1⁺ cells obtained from ROSA26 mice were used as donor cells. Mice were sacrificed with a lethal inhalation of halothane followed by decapitation. Brains were quickly extracted and fixed for 30 min at RT in a mix of 0.2% glutaraldehyde and 1.48% formaldehyde in PBS. Brains were then sliced into 1 mm-thick slices and β-galactosidase expressing cells were detected by incubating the sections at 37°C overnight with X-gal (final concentration of 1 mg/ml) in X-Gal developer mix (35mM K₃Fe(CN)₆/35mM K₄Fe(CN)₆·3H₂O/2mM MgCl₂ in PBS) to form a blue reaction product within the cell. Slices were then incubated in 20% sucrose overnight, then frozen and processed for cryosectioning. From each 1mm brain slice 10 µm sections separated by 50µm were obtained using a cryotome and counterstained with nuclear fast red resulting in a pink background. Cell counting was done with an automated Zeiss microscope and was fully computerized with the combined use of two advanced softwares that are directly connected to the microscope: the AxioVision Rel4.5 and the GSA Image Analyzer

v3.5.6 softwares: the AxioVision Rel4.5 allowed precise and complete automated scan of consecutive and adjacent fields for full coverage of the slices and image acquisition; the acquired images were directly relayed to the GSA Image Analyzer v3.5.6 which counted the cells. The LacZ+ cells and total brain cells in each field were electronically counted based on morphological characteristics recognized by the software. The amount of LacZ+ cells was then corrected to the total number of cells on the slices used, and expressed as number of LacZ+ cells per 10000 total brain cells.

Immunohistochemistry—Frozen 10 μ m brain sections were processed for the detection of neurons and astrocytes using the NeuN and GFAP markers, respectively. Briefly, cells were permeabilized with 0.2% Triton X-100 for 30 minutes at room temperature (RT), washed with PBS and blocked with 3% Normal Goat Serum (Vector Laboratories), then incubated with primary antibodies at 4°C overnight (mouse anti-NeuN, 1:200, Chemicon; rabbit anti-GFAP, 1:200, Santa Cruz). Sections were then washed with 0.1% Triton X-100 and incubated with secondary antibodies (goat anti-mouse Fluorescein, 1:200; goat anti-rabbit Texas Red 1:200, both from Chemicon) for 2.5h at RT, washed with 0.1% Triton X-100, then DAPI was added. Sections were examined using a Zeiss fluorescent microscope, and pictures were acquired using AxioCan M Rm camera (Zeiss) and AxioVision Rel4.5 software. The quantification of the cells was done as described above for LacZ cells, and the relative proportion of NeuN+ cells and GFAP+ cells were expressed as percentage relative to the total number of DAPI+ cells counted.

Statistical analysis—Statistical analysis was performed using the Student t-test available in Microsoft Excel Software, and values were considered significant when $p < 0.05$.

Results

Generating the MCAO model: infarct size, survival, and behavior

Permanent occlusion of the middle cerebral artery resulted in death of the mice within 4 days ($n=10$; Table 1). The infarct was substantial and occupied most of the affected hemisphere after 24h and 72h occlusion (Figure 1A). Occlusion for 2h ($n = 12$) and 4h ($n = 7$) resulted in the reproducible formation of a large infarct (infarct volume occupied $67.3 \pm 8.4\%$ and $65.2 \pm 7.1\%$ of the ipsilateral hemisphere after 2h- and 4h-occlusion, respectively) that increased in intensity with the occlusion time when brains were stained immediately after reperfusion (Figure 1B). When mice were subjected to a two-hour occlusion followed by reperfusion ($n=115$), the survival time was < 2 weeks (only one mouse survived past 2 weeks) (Table 2), and TTC staining of their brains at various times after reperfusion (16h, 24h, 72h) revealed complete tissue death by 16h, while the infarct size remained comparable overtime (Infarct volume relative to the ipsilateral hemisphere, respectively: $65.56 \pm 7.57\%$, $n=3$; $71.15 \pm 7.82\%$, $n=6$; $67.34 \pm 9.13\%$, $n=4$) (Figure 1C).

Mice subjected to 2h-occlusion followed by reperfusion were further characterized to assess the neurological impairment (Table 4). They all displayed a circular clockwise motion, torso turned towards the right, inability to bear their weight on the side of paralysis, impaired righting reflex. 29% of them displayed barrel rolling precipitated by either partially lifting the mouse by the tail, or placing it on its back. Due to the reproducibility of the phenotype it yielded, a 2h occlusion followed by reperfusion was adopted as our model in this study.

Bone marrow transplantation (BMT) strategy and findings

In order to test the effect of cellular therapy on the phenotype of our stroke model, we first used total bone marrow cell infusion, with bulk unpurified BM cells as donor, by exploring three experimental parameters: number of total bone marrow (BM) cells injected (7.0×10^7 ,

1.5×10^8 , 2.0×10^8 , 5.0×10^8), number of injections (1, 2 or 3), and timing of cell administration (same day of surgery or day 1, following days: day 2, 3, 4). Animals subjected to MCAO and injected with PBS were used as controls (n=13). They all showed the same phenotype and survival as the non-injected MCAO mice (data not shown).

Animals subjected to MCAO received total bone marrow cells in one injection on day 1 (7.0×10^7 cells; n=3) or day 2 (7.0×10^7 cells, n=6; 1.5×10^8 cells, n=6), in 2 equal injections on days 1 and 2 (total 2.0×10^8 cells; n=3), or in 3 equal injections on days 2, 3, 4 (5.0×10^8 cells; n=6). (Table 3). Mice transplanted on day 1 all died within the following 24h while mice transplanted on day 2 with an equal number of cells showed a slightly better pattern with one mouse remaining alive for over two weeks and two surviving 4 days. Injection of twice more cells on day 2 allowed few mice to survive 5 or 7 days while the remaining died within 24h (Table 3). When cells were injected in two equal doses on day 1 & 2, none of them died in 24h, and two of three mice survived 6 and 7 days respectively. BMT in three injections at days 2, 3 & 4 of 5.0×10^8 cells did not show any improvement in survival compared to mice transplanted on days 1 & 2 (Table 3). Although most of these mice died within the range of non-transplanted controls, there was a clear shift towards longer survival when more cells were used (7.0×10^7 vs. 1.5×10^8 & 5.0×10^8). The timing of cell injection also seemed to be important: three consecutive injections on days 2, 3 & 4 were not more efficient than two injections on days 1 & 2. We therefore hypothesized that the best BMT strategy would be two injections on days 1 (day of surgery) & 2 (one day after surgery). The data also suggested that a very large amount of cells does not seem necessary or beneficial. Furthermore, most mice which were transplanted with total unpurified BM cells died in less than 2 weeks even with higher amounts of cells, suggesting the possibility that inflammatory cells present in the total BM might be having a negative effect. Therefore we decided to use an enriched population of HSC where differentiated “lineage” cells would be removed.

Sca1⁺ selection provides over 6-fold enrichment of stem and progenitor cells

Total mouse bone marrow cells obtained from healthy donors were subjected to lineage depletion through Sca1⁺-selection with Phycoerythrin-labelled Sca1 antibody.

Flow cytometry analysis of bone marrow cells before and after enrichment showed that the percentage of Sca1⁺ cells increased from $12.9 \pm 1.1\%$ before selection to $81.9 \pm 10.5\%$ after selection (n=5) (Figure 2A), a 6.4 ± 0.7 fold enrichment (Figure 2B). These data provided direct evidence for elimination of a major part of lineage (differentiated and inflammatory) cells.

Sca1⁺ bone marrow cell transplantation rescues mice after MCAO

In order to test if HSCT can rescue the phenotype of mice in our model, Sca1⁺-enriched bone marrow cell population were collected from healthy C57BL/6 and intravenously administered to MCAO mice ~2h after the reperfusion. Increasing number of cells (5.0×10^5 , n=7; 1.0×10^6 , n=3; 1.5×10^6 , n=4; 2.0×10^6 , n=4; 3.0×10^6 , n=16) resulted in a modest increase in survival (Figure 3A). However, the majority of mice died. When mice were transplanted with two doses of 3.0×10^6 cells with the second dose 24h later (totaling 6.0×10^6 cells, n=29), ~40% of mice survived >2 months (Figure 3A). A similar result was obtained after doubling the number of cells per injection to 6.0×10^6 , totaling 12×10^6 cells in two doses (n=11) (Figure 3A). Kaplan-Meier survival curves illustrate the improvement of survival for these mice (Figure 3B).

Surviving mice had smaller infarct and improved behavior

Surviving mice exhibited a rapid improvement in the behavioral phenotype, with the righting reflex, barrel rolling and ability to bare weight corrected within a week after the transplantation, and the circular motion and the torso turning to the right substantially reduced after a week and

fully corrected by 14 days after the transplantation (Table 4). When the brains of transplant-recipient mice were dissected at day 5 after HSCT and compared to age-matched controls, the infarct size was much smaller in animals receiving transplantation (infarct volume relative to the ipsilateral hemisphere ranged from 8.22% to 29.79% in transplant-recipient mice, compared to 65.07% to 75.33% in non-transplanted MCAO controls) (Figure 4A, 4C), indicating an effect of donor HSC on brain tissue recovery.

When brains of transplant-recipient mice were analyzed 5 months after the transplantation, no infarct was detected using TTC staining (Figure 4B, panels a, b) demonstrating complete clearance of the dead tissue. Shrinkage of the affected hemisphere was observed (Figure 4B) but the missing tissue portion (7.76% to 15.38% of the ipsilateral hemisphere) was much smaller than the original infarct size that averaged 69.7% (Figure 4B panels a, b; Figure 4C), rendering difficult even to notice the difference in brain size in certain animals (Figure 4B, panels c, d). Such observation strongly suggests the presence of a protective effect from loss of tissue.

Tracking analysis revealed little donor cells in recipient brains and differences between various recipients

To verify whether donor cells have reached the brain following IV injection, Scd1+ cell transplantations were performed with donor cells obtained from ROSA26 on C57BL/6J background. Analysis of recipient brains (n=10) revealed the presence of LacZ+ cells (Figure 5), suggesting that donor cells have crossed the blood-brain barrier (BBB). These cells were observed in a narrow zone of cortical and subcortical areas, which roughly correspond to the peripheral part of the infarct. However, their density was very low (Figure 5C & D), and quantifications showed ~ 1.4–4.7 LacZ+ cells per 10 000 brain cells (Figure 5E), suggesting that their effect is unlikely to be through cell replacement/supplementation.

Long term examination of brains revealed neuronal loss and increased astrocytes

Five months after the MCAO, transplant-recipient mice were sacrificed and brains processed for the detection of neurons using NeuN marker. Our data showed 23% less neurons in the ipsilateral hemisphere relative to the contra-lateral hemisphere. There was also a parallel increase in the proportion of GFAP+ cells, which, on average, were ~61% higher in the ipsilateral hemisphere (Figure 5, F & G). These data suggest long term loss of some neurons and an astrocytosis in surviving mice.

Discussion

Stroke often results in high morbidity and mortality. To better understand the deleterious effects of blood vessel occlusion and improve potential stem cell treatment in humans, we have used a mouse stroke model. With permanent occlusion, a large brain lesion was detected (Figure 1A), but all mice died within 4 days (Table 1). We then reasoned that a transient occlusion would be a better model. We therefore performed MCAO with reperfusion and stained the brains with TTC either immediately after reperfusion or at various times afterwards. We found that after 2h occlusion, the infarct size was reproducible and large enough to be used for assessing any transplantation induced recovery (Figure 1B, 1C). These results suggested that reperfusion after 2h occlusion results in a slower propagation of the brain damage, allowing the mice to live longer, working as reproducible model for future therapeutic protocols.

To determine whether stem cell transplantation can improve infarct size and survival of mice, we first chose murine total bone marrow cells to conduct the experiments. These cells contain at least three types of stem cells: hematopoietic, mesenchymal and endothelial, which could provide a combinatory effect. We reasoned that injecting cells intravenously will pose no

additional damage to the brain tissue, contrary to intra-brain injection. The survival rate was slightly prolonged when larger amounts of cells were administered. Two injections on days 1 and 2 had a better survival profile than a single injection on either day 1 or 2. Three injections on days 2, 3 & 4 did not improve survival protection. Altogether, these data suggested that two injections would be more efficient than a single one. However, the fact that even large amounts of cells failed to prolong the survival of mice beyond 2 weeks suggested a possible deleterious effect of inflammatory cells contained in the total bone marrow cell mix. Therefore we chose to use a cell population that is enriched in murine hematopoietic stem and progenitor cells, obtained by a positive selection using the Sca1 cell surface marker (Sca1⁺-population). Sca1 is expressed on the surface of hematopoietic stem cells and early progenitor cells but not on lineage positive cells (Wilson et al, 2007). The selection for Sca1⁺ cells provides a substantial enrichment in stem cells (Figure 2). This cell population is also depleted of lineage (differentiated) cells which could potentially be harmful. The enriched population of stem and respective progenitors can be safely administered and are readily available. Sca1⁺-enriched cells have not been previously tested in this particular setting. Starting with a low cell dose (5×10^5 cells) and progressively increasing the dose increased the survival rate of transplant-recipient mice. This data suggests a positive effect of donor cells on animal protection and culminates in ~40% survival with 6 million Sca1⁺ cells in two injections (Figures 3A, 3B). Since mice did not survive after receiving only one injection at either day 1 or day 2, but survived after 2 consecutive injections at days 1 & 2, we hypothesize that the first injection partially stabilizes the mice while the second functions as a booster, providing additional protection resulting in the observed recovery. The finding that the amount of brain tissue lost (at 5 months post-transplantation) was much smaller than the infarct size generated by the MCAO procedure used, (Figure 4A) implies that donor stem cells rescued the mice by protecting the brain tissue.

Tracking analysis revealed only very few donor cells in the brains of the recipients indicating that cell supplementation of ischemic brain by donor cells is unlikely to account for the recovery observed (Figure 5). The quantification of these cells helped us divide the recipient mice into two groups one with lower number (<2/10 000) and one with higher number (3–5/10 000) of LacZ⁺ cells (Figure 5E). Although in general the number of LacZ⁺ cells detected was low, these results suggested that the contribution of donor cells to the recovery might have depended, at least in part, on the number reaching the brain, with higher numbers providing more efficient protection, and could explain, partially, why recovery was observed in some but not all mice. The long-term (5 months) examination of brains from MCAO mice that received Sca1⁺ cells (Figure 4B) revealed little shrinkage of the affected hemisphere relative to the original infarct size (12.5% \pm 3.3%) (Figure 1C) suggestive of some protection of brain tissue. Our immunohistochemistry examination of neurons in surviving mice 5 months after HSCT showed that they were significantly ($p < 0.03$) lower than controls in parallel to an increased astrocyte proportion, suggesting that the neuroprotective effect of HSCT that rescued these mice did not save the entire neuronal population. Given the shrinkage of the ipsilateral hemisphere and the loss of almost one fifth of the neuronal population, it is likely that the tissue loss was mostly due to the neuronal loss. The increase in the non-neuron cells might have prevented major tissue loss and could have contributed to the protection as well. Since the amount of brain tissue lost in transplant-recipient mice was much smaller than the size of the infarct observed after the stroke alone, the HSCT is very likely to have contributed to protecting a major proportion of brain cells. Although the molecular mechanism of this rescue remains to be elucidated, a previous study had demonstrated that ischemic brain extracts can induce growth factor secretion by human BM mesenchymal stem cells (Chen et al, 2002). It is tempting to speculate that a similar effect might also occur with murine HSC. Donor cells might secrete soluble protective factors not only in the brain, but also while in the circulation within the first few hours after injection. Since the blood-brain barrier (BBB) is impaired after stroke (Dijkhuizen et al, 2002; Montaner et al, 2003), some of these factors might cross the BBB and

contribute to the recovery by helping endogenous cells survive. In addition, hMSC have been shown to modulate the inflammatory reaction in the brain (Ohtaki et al, 2008). Altogether, these data suggest that a reciprocal interaction between donor cells and the ischemic brain tissue might play a role in the recovery. We speculate that this induction could follow the trend of a positive feed-back loop between donor and recipient cells, whereby the ischemic tissue releases cytokines that stimulates donor cells to secrete growth and neurotrophic factors which in turn protect recipient cells. Future studies will help shedding the light on various aspects of the mechanism by which transplanted Sca1+ cells provide their neuroprotective effect.

In summary, we have demonstrated for the first time the ability of murine Sca1+ bone marrow cells to rescue 40% of mice subjected to lethal stroke through HSCT, reducing the infarct size and neurological deficit. We also showed that timing of cell injection has important effects on the above outcome. These results may have important implications on future human stroke, using a stem cell therapy.

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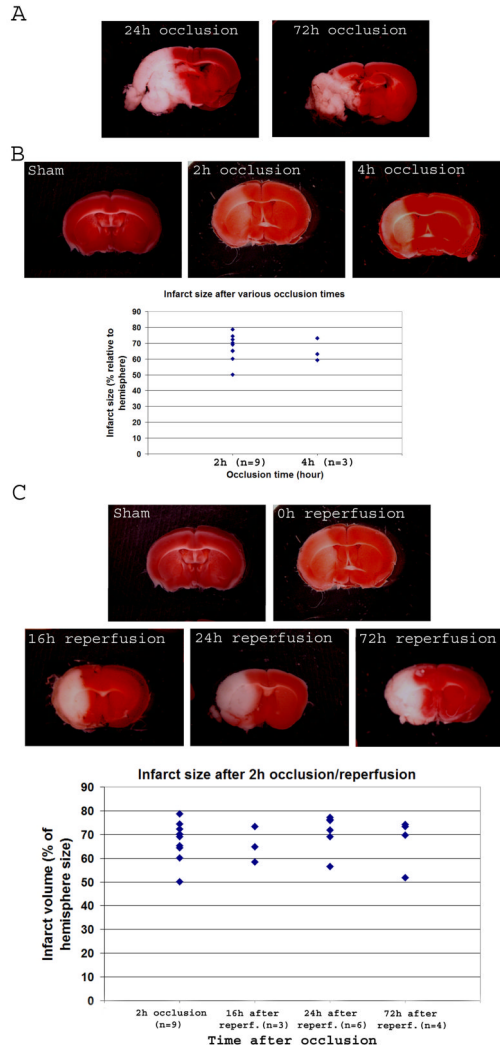
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GENERATING THE STROKE MODEL

**FIGURE 1. Generating the stroke model**

Brains of control mice and mice subjected to stroke were sectioned and stained with 2, 3, 5 triphenyl tetrazolium chloride (TTC) to detect the infarct area. Intact tissue stained red while infarcted tissue appears white.

1A-After permanent occlusion, TTC-stained brains after 24h and 72h of MCAO revealed a major infarct area occupying most of the hemisphere, and liquefying at 72h.

1B-Permanent occlusion of only 2h or 4h resulted in a clear infarct, more severe with a longer occlusion time, and respectively occupying $69.7 \pm 6.5\%$ and $65.2 \pm 7.1\%$ of the ipsilateral hemisphere as shown in the histogram.

1C-Mice were subjected to 2h MCAO followed by reperfusion and TTC staining at variable times afterwards (16h, 24h, 72h). The infarct area appeared a mix of white and pink in color just after 2h. Overtime, the color became fully white in the affected area suggesting complete death of the tissue in the affected area. The scatter graphic shows a comparative quantification of the infarct size just after 2h occlusion (left bar), then at various times after reperfusion of a 2h-occlusion MCAO (16h, 24h and 72h after reperfusion, respectively second, third and fourth cluster).

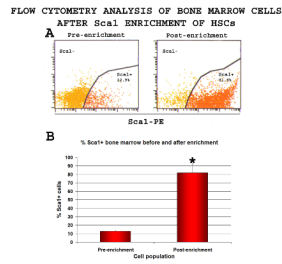


Figure 2. Sca1 positive selection of hematopoietic stem and progenitor cells

Total bone marrow cells were labeled with Phycoerythrin-coupled anti-Sca1 antibody and processed for positive selection of Sca1+ cells. Samples taken before and after enrichment were analyzed.

A-The Sca1+ cell population increased from $12.9 \pm 1.1\%$ before enrichment to $81.9 \pm 10.5\%$ after enrichment (n=5)

B-A 6.4 ± 0.7 fold increase in the percentage of Sca1+ cells was observed (n=5)

*T-test, % Sca1+ cells before enrichment vs. after enrichment, $p < 0.0001$.

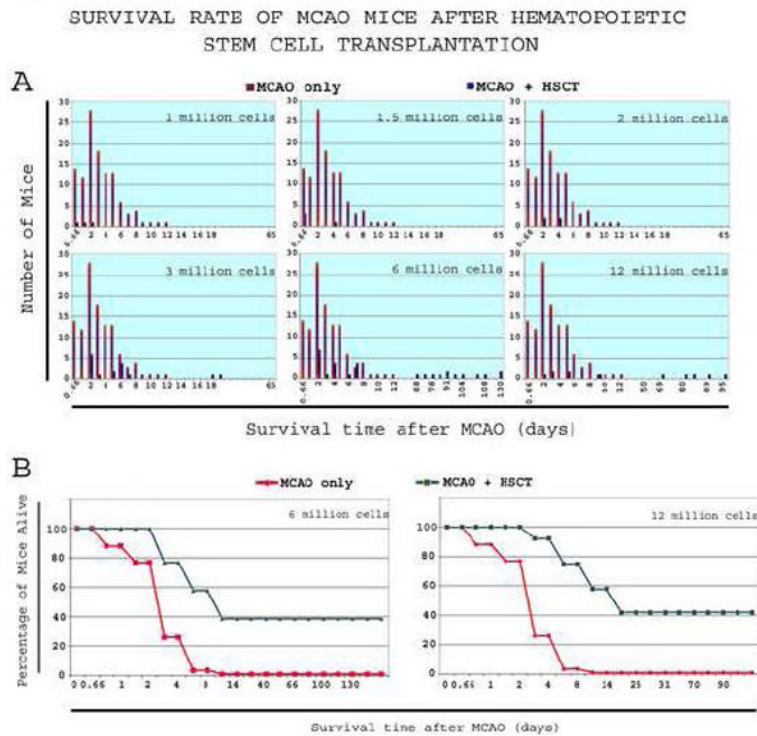


Figure 3. Survival rate of MCAO mice after Sca1+ bone marrow cell transplantation
Mice subjected to MCAO followed by Sca1+ cell transplantation, as well as non-transplanted control, had their lifespan measured

3A-Over 60% of the controls died within 3 days. After Sca1+ cell transplantation, a progressive shift-to-the-right in survival was observed with increasing number of cells. Starting with 2 million cells, no mice died in <24h, and with 3 million cells, the average survival was clearly shifted towards longer lifespan, with few mice remaining alive after 2 months. Starting 6 million cells, ~40% of mice survived past 2 months.

3B-Kaplan-Meier curves of MCAO controls and transplant-recipient of 3 and 6 million cells shows long-term survival of a relatively large proportion of mice compared to controls.

RESCUE OF BRAIN TISSUE AFTER HEMATOPOIETIC
STEM CELL TRANSPLANTATION (HSCT)

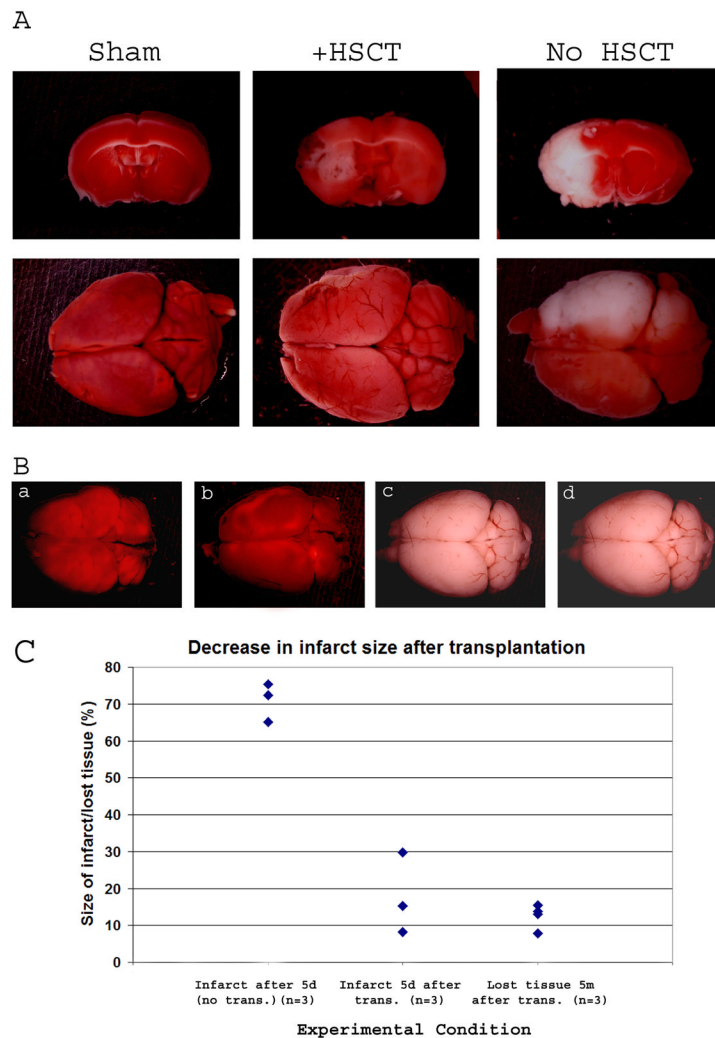


Figure 4. Rescue of brain tissue after Sca1+ bone marrow cell transplantation (HSCT)

At various times after Sca1+ cell transplantation, MCAO mice were sacrificed and their TTC-stained brains were used to evaluate the infarct.

4A-5 days after 2h transient occlusion, the infarct had a volume of $68.7\% \pm 5.15\%$ of the affected hemisphere (right column) and became fully white, while transplant-recipient mice (middle column) showed a clear regression of the infarct size to almost one-half to one-fourth ($17.73\% \pm 11.00\%$).

4B-Transplant-recipient MCAO mice surviving past 2 months after the MCAO were sacrificed at 5 months after the transplantation. TTC Staining did not reveal any infarcted region, suggesting an elimination of the dead tissue overtime. The gross morphology of the brains revealed shrinkage of the affected hemisphere, clearly noticeable in 50% of animals analyzed, as shown in panels a and b. The amount of brain tissue lost ranged from 7.76% to 15.38% of ipsilateral hemisphere.

4C-Quantification of the infarct size shown in panels 4A and 4B. The left cluster represents the infarct size in MCAO controls 5 days after 2h occlusion, the middle cluster represents the infarct size in transplant-recipient (6 million Sca1+ cells) MCAO mice 5 days after a 2h

occlusion, and the right cluster represents the percentage of lost brain tissue from the ipsilateral hemisphere 5 months after Sca1+ transplantation into 2h-occlusion MCAO mice.
Abbreviations: d, day; m, month; trans., transplantation

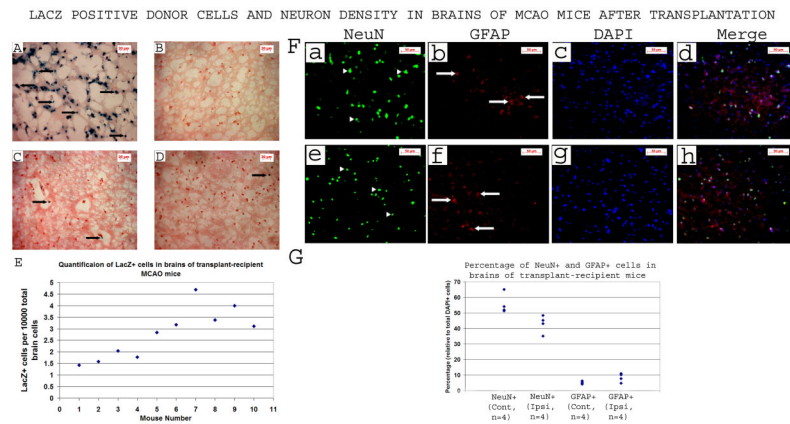


Figure 5. LacZ positive donor cells and neuron density in brains of MCAO mice after transplantation

HSCs from ROSA26 mice were transplanted into MCAO mice in 2 IV injections 1h and 18h after MCAO. 3h after the second injection, mice were sacrificed and brain slices were stained for LacZ (in blue, arrows) and counterstained with nuclear fast red (in red). Only very few donor cells were detected.

A-Positive control for X-Gal staining (ROSA26 mouse brain): LacZ+ cells are readily detectable as blue/black in color (arrows point to some of them).

B-Negative control for X-Gal staining (C57BL/6J mouse): no detectable LacZ+ cells (absence of blue/black cells)

C, D-Brain sections of two separate transplant-recipient MCAO mice: very few LacZ+ cells are detectable (arrows)

E-Quantification of the number of LacZ+ cells in brains of recipient mice: each point represents an individual mouse. Values are expressed as number of LacZ+ cells per 10 000 brain cells.

F-Immunofluorescence staining of brain slices of transplant-recipient MCAO mice 5 months after the stroke/HSCt, both in the ipsilateral (a–d) and contra-lateral (e–h) hemispheres, detecting neurons (in green) using anti-NeuN antibody, and astrocytes (in red) using anti-GFAP antibody. DAPI was used to stain the nuclei (in blue).

G-Quantification of NeuN+ and GFAP+ cells of mice represented in section “F” above. Cont, contra-lateral; Ipsi, ipsilateral. The NeuN+ cell density was lower and the GFAP+ cell density was higher in the ipsilateral hemisphere.

Table 1

Survival time of mice after permanent Middle Cerebral Artery Occlusion

Survival time after MCAO	n
16h	1
1 day	3
2 days	1
3 days	3
4 days	2

A total of 10 mice subjected to permanent MCAO were observed until death occurred, and the time elapsed between the surgery and death was recorded (left column); the “n” above represents the number of mice among the 10 observed that died at each time point indicated in the left column.

Table 2

Survival time of mice after transient middle cerebral artery occlusion followed by reperfusion

Occlusion time	n	Survival
2h	115	16h – 12 days*

n, number of mice; h, hours

* one mouse remained alive all through follow-up

Table 3

Transplantations with total bone marrow cells: strategies and outcomes

Transplantation time	Total No. of cells	No. of injections	No. of recipients	No. of mice surviving > 2 weeks	Survival time of individual mice dying in < 2 weeks
Day 1*	7.0×10^7	1	3	0	< 24h
Day 2	7.0×10^7	1	7	1	< 24h (n=4); 4d (n=2)
Day 2	1.5×10^8	1	7	0	2d (n=4); 5d (n=2); 7d (n=1)
Days 1, 2	2.0×10^8	2	3	0	3d (n=1); 6d (n=1); 7d (n=1)
Days 2, 3, 4	5.0×10^8	3	6	0	2d (n=1); 5d (n=3); 6d (n=2)

No., number; n, number of mice; d, days;

* day 1 is the same day of the MCAO surgery.

Table 4

Neurological scoring of MCAO mice

Mouse groups	Pre-HSCT (2h after MCAO)						Post-HSCT (7 days after transplantation)						14 d		
	n (%)	Tors Tilt ¹	Circul motion ²	Can't bare weight	Barrel rolling	Right reflex	Score	n (%)	Tors tilt	Circul motion	Can't bare weight	Barrel rolling	Right reflex	Score	Score
MCAO cont (A)	15 (71%)	1	2	3	0	+	6	4 (67%)	1	2	3	0	+	6	n/a
MCAO cont (B)	6 (29%)	1	2	3	4	+	10	2 (33%)	1	2	3	4	+	10	n/a
MCAO + 6 × 10 ⁶ HSCs (C)	17 (59%)	1	2	3	0	+	6	5 (42%)	1*	2**	0	0	-	3 ^{a,c}	0
MCAO + 6 × 10 ⁶ HSCs (D)	12 (42%)	1	2	3	4	+	10	2 (17%)	1*	2**	0	0	-	3 ^{b,d}	0
MCAO + 12 × 10 ⁶ HSCs (E)	7 (70%)	1	2	3	0	+	6	3 (60%)	1*	2**	0	0	-	3 ^{e,f}	0
MCAO + 12 × 10 ⁶ HSCs (F)	3 (30%)	1	2	3	4	+	10	1 (20%)	0	0	0	0	-	0	0

n, number of mice; %, percentage of mice; MCAO, middle cerebral artery occlusion; HSCT, hematopoietic stem cell transplantation; HSCs, hematopoietic stem cells; Naive cont., control mice that were not subjected to MCAO; MCAO cont., control mice subjected to MCAO only; Tors tilt, torso turned towards the right; circul motion, circular motion towards the right; n/a, not applicable (mice were dead by that time); 14d, 14 days post HSCT.

Scoring: all signs but the righting reflex were given a score as previously described with slight modification (McCullogh et al., 2005). Different signs were given a different "grading" number and the total score was the sum of the grades of all the graded signs; 0, sign not detected; 1, 2, 3, 4, sign detected; Righting reflex was noted as impaired (+, i.e. mouse shakes/moves randomly before righting up), or normal (-, mouse right up immediately without any random movement).

Circular motion: mouse turns in a circular fashion instead of walking straight; Can't bare weight, mouse is leaning towards the paralyzed side and cannot hold up its body weight on that side; Barrel rolling: when placed on their back or slightly lifted by the tail, mice roll once or twice like a barrel; righting reflex; mice are placed on their back.

¹ the torso is turned to the right in a pronounced way;

* the torso is slightly turned to the right;

² circular motion 10–21 times per minute;

** circular motion ≤ 5 times per minute.

t-test:

^a group C 2h vs. 7 days, $p < 4 \times 10^{-8}$;

^b group D, 2h vs. 7 days, $p < 0.02$;

^c group A vs. C, 7 days, $p < 0.004$;

^d group D vs. B, 7 days, $p < 0.02$;

^e groups E & F, 2h 7 days, $p < 0.0003$;

^f groups E & F, 7 days vs. groups A & B, 7 days, $p < 0.0008$