Intra-arterial Cell Transplantation Provides Timing-Dependent Cell Distribution and Functional Recovery After Stroke

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Background and Purpose—Intra-arterial cell transplantation offers a novel therapeutic strategy for stroke; however, it remains unclear how the timing of cell administration affects cell distribution, brain repair processes, and functional recovery. Here, we investigate the hypothesis that the timing of cell transplantation changes the behavior of the cell graft and the host environment in a way that affects functional recovery.

Methods—Rats received human mesenchymal stem cells via the internal carotid artery at 1, 4, or 7 days (D1, D4, or D7) after middle cerebral artery occlusion and reperfusion. Animals were euthanized at various time points to assess cell distribution, infiltration of activated microglia, expression of brain-derived neurotrophic factor, reactive astrocytes, angiogenesis, and functional recovery.

Results—Human mesenchymal stem cells were widely distributed both in the peri-infarct and core in D1, and dominantly in the peri-infarct in D4. Very few cells were observed on D7. At day 7 poststroke, microglia activation was significantly suppressed in both the peri-infarct and core in D1, and predominantly in the peri-infarct in D4. At day 21 poststroke, brain-derived neurotrophic factor was widely distributed throughout the peri-infarct in D1 and D4, along with many reactive astrocytes and considerable angiogenesis. Motor function improved earlier in D1 and later in D4, but no recovery was obtained in D7.

Conclusions—Our results indicate that intra-arterial cell transplantation provides timing-dependent cell distribution and poststroke functional recovery via a combination of neuroprotection, reactive astrocyte enhancement, and angiogenesis. (Stroke. 2013;44:XXX-XXX.)

Key Words: intra-arterial transplantation ■ mesenchymal stem cells ■ stroke ■ timing

Stem cell therapy has emerged as a promising treatment strategy for stroke.1–4 However, questions remain, particularly with regard to the therapeutic time window, optimal route of delivery, best source of cells, and the mechanisms of recovery.5–6 Among these, the timing of cell transplantation could be critical to a successful outcome and has not been fully investigated.7–9

Recently, endovascular intra-arterial therapy has greatly enhanced the care of patients undergoing neurosurgery. Several animal studies have shown the efficacy of stem cell transplantation via the carotid arteries,5–16 and a single-blind clinical phase II/II trial has showed safety, feasibility, and biological effects of intra-arterial cell transplantation in patients with stroke.17 Therefore, intra-arterial delivery of stem cells could represent a novel therapeutic strategy. Intra-arterial cell transplantation could address the main problem with intravenous delivery: the dispersal of cells throughout the body, with the result that fewer cells are found in the brain parenchyma.11,18–22 There has been much debate over the best method of delivering cells to the stroke site because the mechanism for the functional recovery seen with stem cell treatment has not been determined.8,19 If the cellular replacement or transendothelial migration of the stem cells is necessary to maximize the functional recovery, then the intra-arterial approach could be a powerful option to effectively deliver stem cells to the selected vascular territory. Given the promising development of endovascular therapy in the field of stroke management, further assessment of the intra-arterial delivery of stem cells is urgently needed.

This study aimed to test (1) whether timing of the intra-arterial cell transplantation affects the behavior of the cell graft and host environment, and (2) whether even delayed intra-arterial delivery may contribute to the recovery. Finally, we assessed the hypothesis that intra-arterial cell transplantation provides timing-dependent cell distribution and poststroke functional recovery.
Methods

Middle Cerebral Artery Occlusion and Reperfusion Model

Animal procedures were approved by the Administrative Panel on Laboratory Animal Care of Nagasaki University. Male Sprague-Dawley rats (280±20 g) were subjected to 75 minutes of middle cerebral artery occlusion and reperfusion with a 4.0 nylon monofilament suture coated with silicone (4039PK10; Doccol) under isoflurane anesthesia. Only the rats showing a Bederson score of 3 points were included in the study.

Cell Transplantation

Before cell transplantation, human mesenchymal stem cells (hMSCs) were dissociated. The cells were detached with 0.25% trypsin-EDTA, and 1×10^6 of the cells were diluted with 300 μL of phosphate-buffered saline. The cell solution was injected via the internal carotid artery through a polyethylene catheter (rat carotid artery catheter; Neuroscience), which was inserted from the distal external carotid artery to the internal carotid artery. The cell suspension was injected very slowly (100 μL/min) along with the antegrade flow from the common carotid artery. All the rats received intraperitoneal injections of cyclosporine-A (10 mg/kg) on the day of stroke and then every 2 days until being euthanized. Animals were divided into 3 groups according to the day of the cell delivery: (1) D1: 1 day after stroke, (2) D4: 4 days after stroke, and (3) D7: 7 days after stroke. Vehicle animals received phosphate-buffered saline 1 day after stroke as the control.

Histological Analysis

All quantification and analysis were performed in a double-blinded manner. Stereological quantification of the distribution hMSC was measured by counting antihuman mitochondria-positive cells in the 3 regions of interest in the peri-infarct and core of the cortex and striatum at 3 hours and 72 hours post-transplantation using WinROOF (Mitani Corporation; Tokyo, Japan). The number of ED-1–positive activated microglia in the same regions of interest was counted at 7 days poststroke. To evaluate the host environment at 21 days poststroke, the number of reactive astrocytes coexpressing Nestin and glial fibrillary acidic protein (GFAP) and the expression of brain-derived neurotrophic factor (BDNF) in the ipsilateral peri-infarct were analyzed.

Figure 1. A and B, Three hours after transplantation, many human mesenchymal stem cells (hMSCs) were distributed throughout both the peri-infarct and the core on D1, whereas on D4 most hMSCs were found in the peri-infarct and only very few hMSCs had reached the brain parenchyma in D7 (n=6; #P=0.09, *P<0.05, unpaired t test with 2-tailed value; scale bar=100 μm). C, The ratio of hMSCs in the peri-infarct to those in the core was significantly higher in D4 than in D1 (n=6; *P<0.05, ***P<0.001, Mann-Whitney test). D, Schema showing distribution of hMSCs 3 hours and 72 hours after transplantation (dots show migrating hMSCs).
using WinROOF. Microvessel analysis also was performed by counting von Willebrand factor–positive lumens in the peri-infarct cortex.

Behavioral Assessment
Behavioral assessment was performed at 0, 1, 4, 7, 14, and 21 days after stroke using the cylinder test to measure forelimb use during vertical exploration.26

Brain Atrophy Evaluation
To assess brain atrophy at 21 days poststroke, coronal sections of the brain (1.0 mm before the bregma) were stained with Cresyl violet, and the ratio of the remaining area (ipsilateral/contralateral) was measured using Image J (version 1.43; National Institutes of Health).

Statistical Analysis
Data are presented as means±SEM. Data were tested for normality and equal standard deviations (SDs) in GraphPad InStat (version 3.10; GraphPad Software) to determine the appropriate statistical test (parametric vs nonparametric). The text and Figure legends describe the statistical tests; unless stated differently, all tests were 2-tailed. Differences were considered statistically significant at P<0.05.

Detailed descriptions of our methods appear in the online-only Data Supplement. These methods include cell culture, matrix metalloproteinase-9 (MMP-9) assay, tissue processing, immunohistochemistry, and the cylinder test.

Results
Distribution of hMSCs Depends on the Timing of Transplantation
After the cell transplantation, hMSCs were widely distributed throughout the ipsilateral hemisphere (Figure 1A and 1B). In D1, many hMSCs were detected in the peri-infarct (cortex: 336±137 cells/mm²; striatum: 268±183 cells/mm²) as well as in the core (cortex: 263±136 cells/mm²; striatum: 302±95 cells/mm²) 3 hours after the transplantation. In D4, on the other hand, hMSCs were detected dominantly in the peri-infarct (cortex: 378±254 cells/mm²; striatum: 232±162 cells/mm²) compared with in the core (cortex: 131±141 cells/mm²; striatum: 64±45 cells/mm²). In D7, very few hMSCs were detected in either area. The difference in the pattern of cell distribution among the groups was even more pronounced 72 hours after the delivery. The ratio of the number of hMSCs in the peri-infarct to that in the core was significantly higher in D4 than in D1 (Figure 1C and 1D).

Timing of Transplantation Affects the Host Inflammatory Response and MMP-9 Expression Within 7 Days of Stroke
Seven days after stroke, many ED1-positive activated microglia were seen to have infiltrated both the peri-infarct and core (Figure 2A, cortex; Figure 2B, striatum). However, the animals receiving hMSCs showed fewer ED1-positive cells. On D1, the number of ED1-positive cells was significantly lower in the core and peri-infarct compared with that in the control (Figure 2C). In D4, in contrast, this effect was observed dominantly in the peri-infarct.

It is well-known that MMPs are possible markers for acute ischemic stroke and MMP-9 expression has been reported to be correlated with blood–brain barrier disruption, vasogenic edema, and also inflammation.27,28 Enzyme-linked immunosorbent assay demonstrated that serum MMP-9 protein levels were upregulated at day 1 poststroke (5.0±1.52 ng/mL), and

![Figure 2. A and B.](http://stroke.ahajournals.org/)

Infiltration of activated microglia (green, (ED-1–positive cells) was significantly suppressed after human mesenchymal stem cells (hMSC) transplantation in the peri-infarct cortex (A) and striatum (B) at 7 days poststroke. Asterisk indicates the ischemic core. Scale bar=100 μm. C. Quantitative analysis shows that the number of ED1-positive cells on day 1 (D1) was significantly lower in the peri-infarct and the core compared with that in the control. In D4, in contrast, this effect was observed dominantly in the peri-infarct (n=6; 1-way ANOVA, Tukey-Kramer multiple comparison test, *P<0.05, **P<0.01 vs control).
then gradually reduced at day 4 (1.94±0.76 ng/mL) and day 7 poststroke (0.07±0.07 ng/mL), which was compatible with a previous study. Interestingly, the value of MMP-9 was significantly lower in the D1 group (0.04±0.04 ng/mL) than in the control at day 4 poststroke (Figure I in the online-only Data Supplement).

**Timing of Transplantation Affects the Host Environment at 21 Days Poststroke**

To investigate the repair process at 21 days poststroke, the distribution of BDNF, one of the trophic factors secreted by hMSCs, was quantified in various areas, including the subventricular zone, peri-infarct striatum, peri-infarct cortex, and corpus callosum (Figure 3A). Interestingly, D1 and D4 showed considerable BDNF expression distributed along the peri-infarct (Figure 3B and 3C), along with Nestin- and GFAP double-positive reactive astrocytes and only faint glial scar formation compared with that in control (Figure II in the online-only Data Supplement). There was no obvious difference in reactive astrocyte and glial scar formation between D1 and D4 (data not shown). The number of microvessels also increased in the peri-infarct cortex in D1 and D4 (Figure 3D). On the border area between core and peri-infarct cortex, some BDNF-positive cells were colabeled with GFAP after hMSC transplantation (Figure IIIA in the online-only Data Supplement). However, few BDNF-positive cells were colabeled with GFAP, and most BDNF-positive cells were colabeled with human cytoplasmic marker STEM121 in the peri-infarct cortex (Figure IIIB in the online-only Data Supplement). On the other hand, many BDNF-positive cells were colabeled with NeuN in the area adjacent to the peri-infarct, which was more obvious in hMSC-treated animals compared with control animals (Figure IIIC in the online-only Data Supplement). No BDNF-positive and STEM121-positive cells or BDNF-positive and GFAP positive cells were detected in the area adjacent to the peri-infarct (data not shown). At this time point, the number of surviving cells was 50 to 100 cells/mm² in D1 and D4, which was approximately an 80% decrease from 3 hours after transplantation (Figure IVA in the online-only Data Supplement). Some human mitochondria-positive hMSCs expressed GFAP or von Willebrand factor, but no neuronal markers (Figure IVB in the online-only Data Supplement).

**Functional Recovery Pattern Differs According to the Timing of Transplantation**

Intra-arterial delivery of hMSCs significantly enhanced functional recovery, as assessed by the cylinder test, at 7, 14, and 21 days poststroke in D1 and D4 (Figure 4A). Interestingly, D1 showed an early recovery at 7 days poststroke, and D4 showed a late recovery at 14 days poststroke. No recovery was obtained in D7. The brain atrophy was assessed by Cresyl violet staining. The ipsilateral hemisphere was severely damaged in the cortex (24.3±3.1%), striatum (37.3±9.3%), and corpus callosum (40.2±1.7%) in the control (Figure 4B and 4C). With hMSC transplantation, only D1 exhibited reduced atrophy in the cortex (33.5±3.6%), striatum (60.2±15.0%), and corpus callosum (59.0±2.1%).
Overall mortality evaluated at 21 days poststroke was 31.3% in stroke only, 40.0% in control, 14.2% in D1, 26.9% in D4, and 30.1% in D7, indicating that there was no adverse effect of intra-arterial transplantation to stroke. Intra-arterial cell transplantation–related mortality, defined as mortality occurring within 48 hours of injection, was 10.1% in D1, 19.2% in D4, and 30.8% in D7 (Table).

**Discussion**

In this study, we provide new evidence that the timing of intra-arterial cell transplantation drastically affects cell distribution and functional recovery after stroke. In D1 transplantation, the cell graft works both in the core and in the peri-infarct, whereas in D4 transplantation, the effect is seen mainly in the peri-infarct. Early neuroprotection and later reactive astrocyte enhancement and angiogenesis were evident, with a wide distribution of BDNF in both D1 and D4, but the point of action of the hMSCs differed between D1 and D4. This might explain the disparity in the resulting patterns of behavior recovery and brain atrophy. In this study, a much higher number of hMSCs migrated into the ipsilateral parenchyma with intra-arterial injection (approximately 300 cells/mm² with 1×10⁶ cells transplant) than with intravenous delivery (approximately 75 cells/mm² with 1×10⁷ cells transplant), as reported by Liu et al.18 Our result with intra-arterial delivery was compatible with those of previous reports,9,12,13 and several groups also showed lower efficacy of endothelial cell migration into the brain with intravenous delivery at 24 hours poststroke (4% migration of MSCs),21 at 4 hours, at 1, 3, 5, and 14 days poststroke (0% migration of umbilical cord blood mononuclear cells),8 or during stroke surgery (0% migration of umbilical cord blood cells).19 Nevertheless, functional recovery was obtained with intravenous delivery, indicating that secretion of trophic factors from stem cells outside the brain contribute to the recovery.8,18,19,21 The debate over the best form of delivery, intra-arterial or intravenous, is still complicated and ongoing. However, it is clear that intra-arterial cell transplantation produces functional recovery probably attributable to the wide distribution of the cells, indicating that the cell graft in the brain contributes to the recovery by secreting trophic factors or by cell replacement. Therefore, the mechanism of recovery could differ between intra-arterial and intravenous delivery. Further assessment is necessary to determine which method of delivery could maximize the recovery, but intra-arterial delivery affords an advantage in its requirement of a smaller number of cell grafts. This is very important in the clinical setting with respect to the preparation of the donor cells, especially when autologous cells are used. This is the first study to address the optimal therapeutic time window for the

| Table. Mortality in Relation to Intra-arterial Transplantation After Stroke |
|-----------------------------------|---------------------------|
| Intra-arterial Injection-Related Mortality (Within 48 h of Injection) | Overall Mortality at 21 Days Poststroke |
| Stroke only (n=16), % | 31.3 |
| Control (n=10), % | 20.0 | 40.0 |
| Day 1 (n=28), % | 10.1 | 14.2 |
| Day 4 (n=26), % | 19.2 | 26.9 |
| Day 7 (n=13), % | 30.8 | 30.1 |
recovery by intra-arterial delivery, and as such has important translational implications for the management of stroke in the clinical setting.

In terms of the timing of transplantation, Rosenblum et al. recently showed that intra-arterial transplantation of neural stem cells 3 days after hypoxia/ischemia resulted in the highest cell engraftment. Interestingly, they demonstrated that the transendothelial migration of transplanted cells into the parenchyma is highly dependent on the interaction of the adhesion molecule vascular cell adhesion molecule-1 and the cell surface integrin CD49d. On the other hand, Boltze et al. reported on the therapeutic time window for intravenous injection of human umbilical cord blood mononuclear cells, and concluded that transplantation within a 72-hour window resulted in functional recovery even in the absence of transplanted cells in the brain parenchyma. In other words, they propose that the therapeutic effects were more likely brought about by the production of trophic factors outside the brain. For this reason, the therapeutic time window should be discussed separately for intra-arterial and intravenous transplantation, although late delivery provides low benefits with both methods. We are the first to show the association between the functional recovery and the timing of intra-arterial transplantation, and also to propose a mechanism for this phenomenon.

Intra-arterial transplantation at 4 days poststroke can still provide a valuable contribution to the recovery. Timing of the transplantation also could affect the host environment because many changes occur in the postischemic cascades over time leading to microglial activation, inflammation, oxidative stress, and endothelial dysfunction.11 Previously, we reported that intraparenchymal cell transplantation promotes endogenous repair processes, such as angiogenesis, blood–brain barrier integrity, dendritic plasticity, and axonal sprouting in the peri-infarct area after stroke.30,31 In this study, we clearly show that D1 transplantation drastically reduced the recruitment of activated microglia both into the peri-infarct and the core, and that this effect was seen only in the peri-infarct in the D4 transplantation. Given that the cell graft distributes widely in the core in D1, this result indicates that the hMSCs prevent the death of host cells, thereby contributing to the reduced inflammatory response. The decreased expression of MMP-9 with hMSC transplantation in this study strongly suggests a neuroprotective effect. It is well-known that stroke induces the activation of astrocytes to prevent immune cell invasion and to induce neurovascular remodeling.32–34 Reactive astrocytes also are reported to show the progenitor markers Nestin, NG2, and Musashi-1 in the peri-infarct area.25,35 Therefore, reactive astrocytes could play a key role in the recovery of function after stroke, although the relation between cell transplantation and reactive astrocytes is yet to be determined.26 In the peri-infarct, the widely distributed BDNF and numerous reactive astrocytes coexpressing GFAP and Nestin contributed to the prevention of glial scar formation in both D1 and D4, thereby enhancing angiogenesis in the chronic stage. It is noteworthy that exogenous BDNF secreted by hMSCs contributed to this change in the peri-infarct area, and also that endogenous BDNF secretion from host neurons was enhanced in the area adjacent to the peri-infarct.

Conclusions

The current study demonstrates that intra-arterial cell transplantation provides timing-dependent cell distribution and poststroke functional recovery via a combination of neuroprotection, reactive astrocyte enhancement, and angiogenesis. It is our hope that incorporation of this strategy into currently available endovascular techniques would enhance the therapeutic outcome after stroke.

Acknowledgments

The authors thank Seigo Ohba and Izumi Asahina, Division of Regenerative Oral Surgery, Unit of Translational Medicine, Nagasaki University, for providing hMSCs. Reiko Yamashita, Department of Neurosurgery, Nagasaki University, and Kyoji Ohyama, Department of Anatomy and Neurobiology, Nagasaki University, for tissue preparation, and Tonya Bliss and Gary K. Steinberg, Department of Neurosurgery, Stanford University, for helpful discussions and comments.

Source of Funding

This work was supported in part by a grant-in-aid for Scientific Research to Dr Horie (#23791611), Dr Ishizaka (#23791609), and by a Life Science Foundation of Japan to Dr Horie.

Disclosures

None.

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Stroke. published online January 29, 2013;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/early/2013/01/29/STROKEAHA.112.677328

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SUPPLEMENTAL MATERIAL

Methods

Cell Culture

Human Mesenchymal stem cells (hMSCs) were obtained by iliac crest aspiration from a 39-year-old healthy male volunteer. The cells were cultured in Eagle's medium (DMEM; GIBCO, USA) supplemented with 10% fetal bovine serum, basic fibroblast growth factor (3ng/ml) and 2% penicillin streptomycin. After incubation at 37 °C in a 5% CO₂ atmosphere for 24 hours, non-adherent cells were removed by replacement of the medium. The adherent layer reached 90% confluence within 10-14 days. Cells were then detached with 0.25% trypsin ethylenediaminetetraacetic acid (trypsin-EDTA; Invitrogen, USA). The plastic adherent hMSCs were split every 5-7 days thereafter to assess cell growth and cell yield. The cells used in the following experiments were harvested after 2 to 3 passages.

Matrix metalloproteinase-9 (MMP-9) assay

Blood samples were obtained from the left ventricle before perfusion fixation at day 1, day 4 and day 7 post-stroke in the control group (n=4 each) and at day 4 post-stroke in D1 transplantation group (n=4). Samples were then centrifuged at 14,000 rpm for 5 min.
The plasma was collected and protease inhibitor added. Each sample was then aliquoted and stored at −80°C for ELISA. Serum samples were diluted two fold with PBS and serum MMP-9 levels were measured using ELISA kit for MMP-9 (USCN Life science Inc. China) according to the manufacturer’s protocol. All samples were assessed in duplicate. The optical density (OD) was determined using a microplate reader set to 450 nm, and standard curves were generated (The detection range of our assay is from 0.78 to 50 ng/ml).

**Tissue Processing and Immunohistochemistry**

Animals were perfused and fixed with 4% paraformaldehyde and then coronal sections were prepared for Cresyl Violet, immunohistochemical, and immunofluorescence staining. For diaminobenzidine peroxidase staining, sections were incubated with primary antibodies (mouse anti-human mitochondria: MAB1273; Chemicon, USA; 1: 200 and chicken anti-brain derived neurotrophic factor (BDNF): CH15000; Neuromics, USA; 1: 200) overnight for three nights at 4°C. They were then incubated with secondary antibodies (biotinylated Donkey Anti-Chicken IgY: Gallus, USA; 1:500, and avidin-biotin-peroxidase complex kit: ChemMate ENVISION kit/HRP; Dako, Japan) following the antigen retrieval method using target retrieval
solution (pH 6.0; Dako, Carpinteria, CA) for 2 hours at room temperature. The secondary antibodies were visualized with 3, 30-diaminobenzidine.

For the immunofluorescence staining, sections were incubated with primary antibodies (mouse anti-human mitochondria: 1:200, mouse anti-human cytoplasmic marker STEM 121: AB-121-U-050; Stem Cells Inc, USA; 1: 200, mouse anti-ED1: ab31630; Abcam, USA; 1:100, rabbit anti- BDNF: ab1779; Chemicon, 1:500, mouse anti-NeuN: MAB377; Millipore, USA; 1: 200, rabbit anti-von Willebrand factor: vWF; ab6994; Abcam, Tokyo; 1:400, rabbit anti-glial fibrillary acidic protein: GFAP; Z0334; Dako, 1:600, mouse anti-GFAP; MAB3402; Millipore, 1:500, and mouse anti-rat Nestin: MAB353; Chemicon, 1:200) were incubated overnight at 4°C. They were then incubated with secondary antibodies (TOPRO-3: Molecular Probes, 1:1000; Alexa Fluor 488 donkey anti-mouse IgG: Molecular Probes, 1:250; and Alexa Fluor 568 goat anti-rabbit IgG: Molecular Probes, USA; 1:250) for 2 hours at room temperature. The sections were analyzed using a confocal laser microscope (LSM5 Pascal Ver3.2; Zeiss).

**Cylinder test**

Animals were placed in a plexiglas cylinder, and the number of times each rat reared and touched the cylinder in a weight-bearing fashion with the left, right, or both
forelimbs was counted. Approximately 20 of these limb-use movements were counted per trial. The behavior score was calculated using the equation (affected limb use + both limb use)/(unaffected limb use + both limb use), giving a ratio of affected to unaffected limb use.

References

Enzyme-Linked Immunosorbent Assay demonstrated that serum MMP-9 protein levels were up-regulated at day 1 post-stroke, and then gradually reduced at day 4 and day 7 post-stroke. Interestingly, the value of MMP-9 was significantly lower in D1 group than that in control at day 4 post-stroke (n=4, One-Way ANOVA, Tukey-Kramer multiple comparison test, **p<0.01 vs. Control day 1, †p<0.05 vs. Control day 4).
Reactive astrocytes co-expressing Nestin (Green) and GFAP (red) are widely distributed in the periinfarct cortex in the hMSC-treated animals, with a distinct glial scar (dotted circle) at 21 days post-stroke. Scale bar= 200 μm (50 μm in magnified image). Asterisk indicates the ischemic core.
On the border area between core and periinfarct cortex, some BDNF (red) positive cells were co-labeled with GFAP (green) at 21 days post-stroke after hMSC transplantation (A). However, few BDNF (red) positive cells were co-labeled with GFAP (green) and most BDNF (red) positive cells were co-labeled with human cytoplasmic marker STEM 121 (green) in the periinfarct cortex (A, B). On the other hand, many BDNF (red) positive cells were co-labeled with NeuN (green) in the area adjacent to the periinfarct,
which was more obvious in hMSC treated animals compared with control animals (C).

Scale bar= 50 μm. Asterisk indicates the ischemic core.
Supplemental Figure S4

A: Time course of the number of hMSCs (cell survival) in the periinfarct area. B:

Human Mitochondria-positive hMSCs expressing GFAP (arrow, red in magnified
window) or VWF (double arrows). No hMSCs expressing neuronal markers were noted
(data not shown). Asterisk indicates the ischemic core.