Title	Intracerebral, but not intravenous, transplantation of bone marrow stromal cells enhances functional recovery in rat cerebral infarct : An optical imaging study
Author(s)	Kawabori, Masahito; Kuroda, Satoshi; Sugiyama, Taku; Ito, Masaki; Shichinohe, Hideo; Houkin, Kiyohiro; Kuge, Yuji; Tamaki, Nagara
Citation	Neuropathology, 32(3), 217-226 https://doi.org/10.1111/j.1440-1789.2011.01260.x
Issue Date	2012-06
Doc URL	http://hdl.handle.net/2115/70795
Rights	This is the peer reviewed version of the following article: Kawabori, M., Kuroda, S., Sugiyama, T., Ito, M., Shichinohe, H., Houkin, K., Kuge, Y. and Tamaki, N. (2012), Intracerebral, but not intravenous, transplantation of bone marrow stromal cells enhances functional recovery in rat cerebral infarct: An optical imaging study. Neuropathology, 32: 217-226., which has been published in final form at https://doi.org/10.1111/j.1440-1789.2011.01260.x . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
Type	article (author version)
File Information	Neuropathology32_217.pdf

Instructions for use

ABSTRACT

Background – Recent studies have indicated that bone marrow stromal cells (BMSC) may improve neurological function when transplanted into animal model of central nervous system (CNS) disorders including cerebral infarct. However, there are few studies that evaluate the therapeutic benefits of direct and intravenous BMSC transplantation for cerebral infarct.

Objective –This study was aimed to clarify the favorable route of cell delivery for cerebral infarct in rats.

Methods – The rats were subjected to permanent middle cerebral artery occlusion. The BMSC were labeled with near infrared (NIR)-emitting quantum dots, and were transplanted directly (1×10^7 cells) or intravenously (3×10^7 cells) at 7 days after the insult. Using *in vivo* NIR fluorescence imaging technique, the behaviors of BMSC were serially visualized during 4 weeks after transplantation. Motor function was also assessed. Immunohistochemistry was performed to evaluate the fate of the engrafted BMSC.

Results – Direct, but not intravenous, transplantation of BMSC significantly enhanced functional recovery. *In vivo* NIR fluorescence imaging could clearly visualize their migration towards cerebral infarct during 4 weeks after transplantation in direct group, but not in intravenous, group. The BMSC were widely distributed in the ischemic brain and some of them expressed neural cell markers in the direct group, but not in the intravenous group.

Conclusion – These findings strongly suggest that intravenous administration of BMSC has limited effectiveness at clinically relevant timing and direct administration should be chosen for patients with ischemic stroke, although further studies would be warranted to establish the treatment protocol.

(241 words)

Running title:

Direct vs. IV BMSC Delivery

Key words:

Bone marrow stromal cell, cerebral infarct, cell delivery, transplantation, optical imaging

The central nervous system (CNS) possesses a limited capacity for tissue regeneration, and functional recovery after CNS disorders such as ischemic stroke and spinal cord injury are generally considered quite difficult. Therefore, cell transplantation therapy has been expected as one of novel therapeutic strategies for these two decades¹. Embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, neural stem/neuronal precursor cells, umbilical cord blood cells, and bone marrow stromal cells (BMSC) have been considered as candidates for the source of cell transplantation therapy. Of these, the BMSC may be the most likely source of donor cells for patients with CNS disorders in clinical situations, because they can be harvested from the patients themselves without ethical and immunological issues. Recently, there is increasing evidence that the BMSC have the capacity to survive, proliferate, migrate toward injured CNS tissue, express the neuronal and astrocytic phenotypes, and enhance functional recovery when transplanted into various kinds of animal models of CNS disorders, including cerebral infarct¹⁻¹⁸. The BMSC can also produce neurotrophic or neuroprotective factors and support the survival of host CNS tissue. Therefore, the BMSC may protect and regenerate the damaged CNS tissue through multiple mechanisms³. In fact, some preliminary clinical trials have been started to assess the efficacy of BMSC transplantation on cerebral infarct¹⁹⁻²¹.

However, it should be reminded that there still exist a several problems to be solved prior to clinical application of BMSC transplantation for CNS disorders. The issues include the optimal timing, dose, and route of BMSC delivery. Although all of them are quite critical to evaluate its therapeutic significance, there are few studies that scientifically determine the most favorable protocol even in animal experiments using BMSC²²⁻²⁵. For example, the BMSC can be transplanted into the injured CNS tissue through direct, intravenous, intra-arterial, or intrathecal route. However, there is only one study that evaluates the therapeutic benefits of direct and intravenous BMSC transplantation for cerebral infarct²³. As recently pointed out by The STEPS Participants, it is essential to determine the most desirable route of cell delivery in order to yield the maximal therapeutic effects prior to clinical application of cell-based therapy²⁶.

Based on these considerations, this study was aimed to clarify the favorable route of BMSC transplantation for cerebral infarct. For this purpose, the BMSC were transplanted directly or intravenously at 7 days after the onset of permanent middle cerebral artery (MCA) occlusion. In addition to behavioral test, noninvasive *in vivo* near infrared (NIR) fluorescence imaging was employed to serially track the transplanted cells. Finally, the distribution and phenotypic fate of the engrafted cells were evaluated with histological analysis.

MATERIALS AND METHODS

Preparation of BMSC

All animal experiments were approved by the Animal Studies Ethical Committee of the Hokkaido

University Graduate School of Medicine. All animals were purchased from CLEA Japan, Inc. The BMSC were isolated under sterile conditions from the 8- to 10-week-old male Sprague-Dawley (SD) rats, as described previously^{16, 18}. Briefly, anesthesia was induced with 3% isoflurane in N₂O/O₂ (70:30) and maintained via spontaneous ventilation with 2% isoflurane in N₂O/O₂ (70:30). The femurs were aseptically dissected. Their both ends were cut and the marrow was extruded with 5 mL of Dulbecco modified Eagle's medium (DMEM, Nissui Company, Tokyo, Japan) containing 10% fetal bovine serum (FBS), 100U/ml penicillin G, and 10% heparin, using a 2.5-mL syringe and a 21-gauge needle. Between 10 and 15 × 10⁶ whole marrow cells were placed in 150-cm² tissue culture flasks that were coated with collagen I (Beckton Dickinson Labware, Bedford, UK) in DMEM/10% FBS. After 24 hr, the non-adherent cells were removed by changing the medium. The culture medium was replaced two to three times a week. When the cells had grown to confluence, they were lifted by 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS). The cells were passed three times for subsequent experiment.

Labeling of Cultured BMSC with Quantum Dots

The cultured BMSC were labeled with QD800 Q-tracker cell labeling kits (CdSeTe/ZnS: <10nm core-shell type, 5 to 10 nm polymer coating; Invitrogen, Hayward, CA, USA), as described before²⁷. Briefly, 1×10⁶ cells in 4-ml DMEM containing 10% FBS were incubated in a 25-cm² tissue culture flask with 2 µl of QD800 reagent A and 2 µl of QD800 reagent B (2 nM). The cells were incubated at 37°C for 15 hr. Following incubation, the cells were washed three times in PBS.

Permanent MCA Occlusion Model

A focal cerebral infarct was induced by permanent occlusion of the right middle cerebral artery (MCA) with temporary occlusion of the bilateral common carotid arteries (CCAs), as described before²⁷. The 8-week-old male SD rats were anesthetized with the above-mentioned condition. Core temperature was maintained between 36.5 and 37.0°C through the procedures. The bilateral CCAs were exposed through a ventral midline incision of the neck. Then, a 1.5-cm vertical skin incision was performed between the right eye and ear. The temporal muscle was scraped from temporal bone, and a 7×7 mm temporal craniotomy was performed, using a small dental drill. To prevent cerebrospinal fluid (CSF) leakage, the dura mater was carefully kept intact, and the right MCA was ligated, using a 10-0 nylon thread through the dura mater. Then, the cranial window was closed with the temporal bone flap. The temporal muscle and skin were sutured with 4-0 nylon threads, respectively. Subsequently, the bilateral CCAs were occluded by surgical microclips for 1 hr. Only animals that circled towards the paretic side after surgery were included in this study²⁸.

Direct or Intravenous BMSC Transplantation

The QD800-labeled BMSC were directly or intravenously transplanted at 7 days after the onset of permanent MCA occlusion. In the direct group (n=8), the QD800-labeled BMSC were stereotactically transplanted into the right striatum, as reported before^{2, 6-8, 12, 18, 29}. Briefly, the animals were anesthetized with the above-mentioned condition. A burr hole was made 3 mm right to the bregma, using a small dental drill. A Hamilton syringe was inserted 6 mm into the brain parenchyma from the surface of the dura mater, and 20 μ l of cell suspension (totally 1×10^6 cells) was introduced into the striatum over a period of 5 min, using an automatic microinjection pump. To prevent CSF leakage, the burr hole was covered with the pericranium, and the skin was sutured with 4-0 nylon threads. In the vehicle group (n=8), 20 μ l of PBS was stereotactically injected into the right striatum with the same procedures.

In the intravenous group (n=8), the QD800-labeled BMSC were intravenously infused through the tail vein. Briefly, the tail vein was exposed and the PE50 polyethylene catheter (0.580-mm inner diameter, 0.965-mm outer diameter; Becton Dickinson) was inserted into the tail vein. Then, 1 ml of cell suspension (totally 3×10^6 cells) was gently infused through the catheter over a period of 3 min. The catheter was removed, and the tail vein was ligated.

Evaluation of Motor Function after Transplantation

To assess the effect of BMSC transplantation on functional recovery, motor function of the animals was semi-quantitatively assessed before and at 7, 14, 21, 28, and 35 days after the onset of ischemia, using a Rotarod treadmill (Model MK-630; Muromachi Kikai Co.). The Rotarod was set to the acceleration mode from 4 to 40 rpm for 3 min. The animals were trained for 3 days prior to this study. The maximum time the animal stayed on the rotarod was recorded for each performance. The two best values of six trials in each animal were used to represent performance^{4, 7, 27}.

***In vivo* NIR Fluorescence Imaging**

To serially track the transplanted BMSC in the living animals, *in vivo* NIR fluorescence imaging was repeated at 7, 14, 21, and 28 days after direct or intravenous BMSC transplantation, as described before²⁷. Briefly, the animals were anesthetized with the above-mentioned condition, and their heads were shaved to avoid light scattering and autofluorescence. Then, they were transferred to the chamber of the IVIS 200 Imaging System (Xenogen Co., Alameda, CA, USA). The fluorescence emitted from QD800 was detected through the scalp and the skull, using an emission filter of 800 nm and excitation filter of 710 nm. Exposure time was set to 5 sec. To calibrate the fluorescence emitted from QD800, the Eppendorf tube containing 5 nM of QD800 in 1 ml of PBS was placed adjacent to the animals. All captured images were analyzed using the Living Image Software (Xenogen Co., Alameda, CA, USA). On the captured images, the 4-mm-diameter region

of interest (ROI) was placed on the right parietal area of animals. Another ROI was set at the whole part of the Eppendorf tube containing the QD800 solution as a reference. Signal intensity in each ROI was expressed as total efficiency (TE) defined as follow:

$$\text{Total efficiency (\%)} = \text{total emission light (photons/seconds)} / \text{total excitation light (photons/seconds)}$$

Then, the intensity of fluorescence emitted from the QD800-labeled BMSC was determined by calculating the ratio of TE in the right parietal area to that in the reference tube.

Histological Analysis

Following decapitation at 28 days after BMSC transplantation, the brain tissue was immersed in 4% paraformaldehyde for two days and embedded in paraffin. The 4- μm -thick coronal sections from three different levels were prepared for subsequent analysis. To evaluate the QD800-labeled BMSC in the brain, each section was observed through 510 nm long-pass filter under a fluorescence microscope (BS51; Olympus, Tokyo, Japan). To semi-quantitatively determine the number of the cells migrated towards the peri-infarct area, 10 regions of interest (ROIs, 100 μm x 100 μm) were placed on the area adjacent to the boundary zone of infarct. The number of QD800-positive cells was expressed by summing up their number in totally 10 ROIs.

To determine the phenotypic fates of the engrafted BMSC, fluorescence immunohistochemistry was performed^{2, 5-7, 16, 17}. Briefly, each section was treated with the mouse monoclonal antibody against GFAP (dilution 1:500, BD Bioscience, Franklin Lakes, NJ, USA) or NeuN (dilution 1:400, Chemicon, Temecula, CA, USA) at 4°C overnight, and was treated with Zenon Alexa Fluor 488 (Molecular Probes Inc, Eugene, OR, USA) at room temperature for 1 hr. The fluorescence emitted was observed through appropriate filter under a fluorescence microscope.

Statistical Analysis

All data were expressed as mean $\pm$ SE. Continuous data were compared by unpaired t-test between two groups, and with one-factor ANOVA followed by post hoc Fisher's PLSD among three groups. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Direct BMSC Transplantation Enhances Functional Recovery

The vehicle or BMSC (1×10^6 cells) was stereotactically transplanted into the ipsilateral striatum at 7 days post-ischemia in the vehicle or direct group, respectively. The BMSC (3×10^6 cells) were intravenously infused at the same timing in the intravenous group. All animals survived throughout the experiment and were used for subsequent analysis. As shown in Fig. 1, all animals exhibited severe neurological deficit prior to vehicle or BMSC transplantation at 7 days post-ischemia. There was no significant difference in motor function among three experimental groups. Subsequently,

stereotactic injection of vehicle did not significantly improve motor function. Likewise, intravenous infusion of BMSC also resulted in no significant effects on motor function. On the other hands, stereotactic transplantation of BMSC significantly enhanced the recovery of motor function. Thus, the animals in the direct group demonstrated significant improvement of motor function at 14 days ($P<0.05$), 21 days ($P<0.01$), and 28 days after BMSC transplantation ($P<0.05$), when compared with the vehicle group. Their motor function was significantly better than that in the intravenously transplanted animals at 14 days, 21 days, and 28 days after transplantation ($P<0.05$).

Direct Transplantation Improves BMSC Engraftment on Optical Imaging

Using *in vivo* NIR fluorescence imaging, the QD800-labeled BMSC were serially tracked at 7, 14, 21, 28 days after BMSC transplantation. Representative findings are shown in Fig. 2. When the BMSC were stereotactically transplanted into the right striatum, *in vivo* NIR fluorescence imaging could identify the fluorescence emitted from the engrafted BMSC in the right parietal area adjacent to cerebral infarct at all time points after transplantation. The intensity of NIR fluorescence signal gradually increased and reached the peak at 21 days after transplantation. The intensity decreased thereafter. Relative total efficiency in the right parietal area was 0.046 ± 0.005 , 0.052 ± 0.030 , 0.050 ± 0.026 , and 0.042 ± 0.019 at 7, 14, 21, and 28 days after transplantation, respectively (Fig. 3).

On the other hands, no significant NIR fluorescence signal could be detected in the head of the animals that underwent intravenous BMSC transplantation. Thus, relative total efficiency in the right parietal area was 0.03 ± 0.001 , 0.025 ± 0.008 , 0.028 ± 0.007 , and 0.023 ± 0.002 at 7, 14, 21, and 28 days after transplantation, respectively. The values were significantly lower in the intravenous group than in the direct group at 14 days ($P<0.05$), 21 days ($P<0.01$), and 28 days post-transplantation ($P<0.05$, Fig. 3).

Direct BMSC Transplantation Increases Their Survival in The Infarct Brain

The distribution of the QD800-positive cells was evaluated in the direct and intravenous groups at 28 days after transplantation on fluorescence microscopy. Fig. 4 summarizes their distribution in both groups. When the QD800-labeled BMSC were stereotactically transplanted into the ipsilateral striatum, the QD800-positive cells were widely distributed in the ipsilateral hemispheres, including the striatum, neocortex, and white matter. Especially, the QD800-positive cells were densely engrafted in the peri-infarct area. Furthermore, some of them were also found in the contralateral hemispheres (Fig. 4A, C). However, when the QD800-labeled BMSC were intravenously transplanted, the distribution of the QD800-positive cells was largely limited. Much fewer QD800-positive cells migrated towards the peri-infarct area, compared with the direct group (Fig. 4B, D). The number of QD800-positive cells at the boundary zone of infarct in the intravenous

group was significantly smaller than in the direct group: $0.1 \pm 0.3 \times 10^2$ and $25.6 \pm 5.6 \times 10^2$ cells, respectively ($P < 0.01$).

To evaluate the fate of the engrafted BMSC in the infarct brain, fluorescence immunohistochemistry was performed at 28 days after BMSC transplantation. In the direct group, a certain subpopulation of the QD800-positive cells was also positive for NeuN and GFAP (Fig. 5). On the other hands, the QD800-positive cells did not express any specific marker for NeuN and GFAP in the intravenous group, although their number itself was quite small.

DISCUSSION

This study clearly demonstrates that the BMSC significantly enhance functional recovery after cerebral infarct in rats, when directly transplanted into the ipsilateral striatum at 7 days after the onset. However, intravenous BMSC transplantation at the same timing did not improve motor function throughout the experiments. *In vivo* NIR optic imaging technique supports these findings. Thus, the technique can non-invasively demonstrate that the NIR fluorescence gradually increases and reaches the peak in the peri-infarct area, when the QD800-labeled cells are directly transplanted into the ipsilateral striatum. On the other hands, no significant NIR fluorescence can be detected under the same condition, when three-folds number of BMSC is intravenously infused. Histological analysis also finds distinct differences between two groups. Thus, the BMSC aggressively migrate into the peri-infarct area and some of them acquire the neuronal and astrocytic phenotypes when directly transplanted into the ipsilateral striatum. However, the number of cells engrafted in the ischemic brain is much fewer in the intravenously transplanted animals. Immunohistochemistry cannot identify their differentiation into the neural cells.

Many of previous studies have suggested that intravenous BMSC transplantation may significantly enhance functional recovery after cerebral infarct, when the BMSC is infused “*within 24 hr*” after the onset^{23,30}. However, therapeutic effects of intravenous BMSC delivery at 1 month post-ischemia still remains to be debated³¹⁻³³. The discrepancy among previous studies may result from the difference in the methodology of neurological assessment. In addition, histological analysis has proven that no or only a small number of the BMSC is engrafted in the host brain when they are intravenously transplanted³⁴⁻³⁶. Therefore, it is most likely that the BMSC protect the host CNS tissue by secreting the neurotrophic factors or by enhancing angiogenesis around cerebral infarct, when they are intravenously transplanted in acute stage of cerebral ischemia³⁷⁻³⁹. Anyhow, the protocol of intravenous transplantation at 24 hr after the onset is not practical from the viewpoints of clinical situations because *in vitro* BMSC expansion requires at least one or two weeks. On the other hands, direct transplantation can deliver the BMSC to the host CNS more efficiently, although it may involve the risk for additional CNS injury. In fact, previous studies have shown that the BMSC significantly promote functional recovery after cerebral infarct when directly

transplanted at 7 days after the onset. Thus, the directly transplanted BMSC aggressively migrate towards cerebral infarct through chemokine systems, and many of them are engrafted in the peri-infarct area^{7,40}. A certain subpopulation of them also acquires the phenotypes of neural cells in the peri-infarct area^{2, 4, 6-8, 27, 29}. As aforementioned, however, there are limited numbers of studies that systematically evaluate the therapeutic benefits of direct and intravenous BMSC transplantation for CNS disorders at clinically relevant timing. Thus, Jendelova et al. (2003) directly injected the BMSC (3×10^5 cells) into the contralateral hemisphere or intravenously injected the BMSC (2×10^6 cells) at 24 hr after the onset of cerebral infarct. They found a massive migration of the engrafted cells into the lesion site regardless of the route of administration²³. Baksi et al. (2004) transplanted the BMSC intravenously, intraventricularly, or intrathecally at 24 hr after the onset of spinal cord injury (SCI) in rats. As the results, they concluded that intraventricular and intrathecal delivery yielded more efficient engraftment of BMSC than intravenous delivery²⁴. Likewise, Paul et al. (2009) recently transplanted the human BMSC directly, intrathecally, or intravenously at 24 hr after the onset of SCI in rats. They found that cell engraftment efficiency was highest after direct delivery and lowest after intravenous delivery²². Lundberg et al. (2009) also transplanted the human BMSC (5 or 25×10^5 cells) into the ipsilateral internal carotid artery or internal jugular vein at 24 hr after the onset of traumatic brain injury in rats, and found a significantly higher number of transplanted cells in the injured hemisphere after intra-arterial than intravenous administration⁴¹. In all of these studies, however, the BMSC were transplanted “at 24 hr” after the insults, and the beneficial effects on motor function was not evaluated. On the other hands, Vaquero et al. (2006) transplanted the BMSC (3×10^6 cells) intravenously or into the injured cord cavity at 3 months after the onset of SCI in rats. They assessed functional recovery during 6 months after transplantation and evaluated histological findings thereafter. As the results, they found that the directly transplanted animals, but not intravenously treated animals, showed a significant functional recovery after SCI. They also concluded that this effect is associated to long-term engraftment of BMSC and their neural differentiation in the injured spinal cord²⁵. Based on these observations, the present study is the first report to clearly denote that direct BMSC delivery is superior to intravenous BMSC delivery in the viewpoints of functional recovery and histological findings, when the BMSC are engrafted “at 7 days” after the onset of cerebral infarct.

In addition, this study also demonstrates that *in vivo* NIR fluorescence imaging can non-invasively distinguish the therapeutic effects of direct and intravenous BMSC transplantation by visualizing the engrafted cells through the skull and scalp. Recent progress in nanotechnology has enabled us to apply biocompatible fluorescence semi-conductor nanocrystal, called as quantum dots (QDs). The QDs have relatively narrow luminescence bands and high resistance to photobleaching⁴². Especially, NIR-emitting QDs are known to emit fluorescence with long

wavelengths (> 700 nm) that allow easy penetration of the tissue, including the bone and skin^{43, 44}. Therefore, *in vivo* NIR fluorescence imaging is expected as a non-invasive modality to serially track the engrafted cells and to validate the therapeutic benefits of cell transplantation therapy in the field of regenerative medicine. In fact, Lin et al. (2007) labeled the ES cells with six different QDs and subcutaneously injected them into the mice back. They found that the QD800-labeled cells provided most prominent fluorescence intensity⁴⁵. Noh et al. (2008) also labeled the dendritic cells with QD800 and subcutaneously injected them into the mice hind limb. They concluded that QD800 labeling had no adverse effects on the biological features of the dendritic cells, and that *in vivo* optical imaging could track their homing into the lymph nodes⁴⁶. Very recently, we have also confirmed that QD800 does not affect the viability of BMSC when the concentration of QD800 in the culture medium was less than 5 nM. We have also succeeded to visualize the QD800-labeled BMSC in the rat brain non-invasively and serially. Thus, *in vivo* NIR fluorescence imaging can serially track the QD800-labeled BMSC over 8 weeks after transplantation, when the cells are directly transplanted into the ipsilateral striatum of the rats subjected to permanent MCA occlusion at 7 days after the insult. The intensity of NIR fluorescence gradually increases in the peri-infarct area, reaches the peak at 3 to 4 weeks after transplantation, and gradually decreases thereafter. Denaturation of QDs and asymmetric division of QDs during cell proliferation most likely contribute to the gradual attenuation of NIR fluorescence detected. The findings on *in vivo* NIR fluorescence imaging correlate very well with those on *ex vivo* NIR fluorescence imaging and histological analysis²⁷. These results are quite similar to the present findings in the animals that are treated with direct BMSC transplantation. Thus, *in vivo* optical imaging can identify NIR fluorescence emitted from the QD800-labeled BMSC when the BMSC are directly transplanted into the ipsilateral striatum, because a significant number of them are engrafted in the peri-infarct neocortex. On the other hands, almost no NIR fluorescence can be detected throughout the experiments, when the BMSC are intravenously injected. The finding correlates well with histological findings. In this study, *in vivo* optical imaging is very valuable to monitor the engraftment of donor cells in the brain and to assess the therapeutic efficacy of each delivery route in the living animals. Thus, it would be essential to establish *in vivo* imaging technique such as optical imaging and MR imaging to objectively evaluate the fate of donor cells in the human brain when applying cell transplantation therapy into clinical situations⁴⁷.

In conclusion, the present study clearly demonstrates that the BMSC significantly promote functional recovery when directly injected into the ipsilateral striatum at 7 days after the onset of permanent MCA occlusion of rats. On the other hands, therapeutic effects are minimal when the BMSC are intravenously delivered at the same timing. This is the first report to scientifically compare the therapeutic effects of BMSC transplantation on cerebral infarct between two different routes of cell delivery at clinically relevant timing. The data strongly suggest that intravenous

administration of BMSC has limited effectiveness and direct administration should be chosen for patients with ischemic stroke, although further studies would be warranted to establish final protocol in clinical situations.

REFERENCES

1. Dharmasaroja P. Bone marrow-derived mesenchymal stem cells for the treatment of ischemic stroke. *J Clin Neurosci*. 2009;16(1):12-20.
2. Shichinohe H, Kuroda S, Yano S, et al. Improved expression of gamma-aminobutyric acid receptor in mice with cerebral infarct and transplanted bone marrow stromal cells: an autoradiographic and histologic analysis. *J Nucl Med*. 2006;47(3):486-491.
3. Hokari M, Kuroda S, Shichinohe H, Yano S, Hida K, Iwasaki Y. Bone marrow stromal cells protect and repair damaged neurons through multiple mechanisms. *J Neurosci Res*. 2008;86(5):1024-1035.
4. Hokari M, Kuroda S, Chiba Y, Maruichi K, Iwasaki Y. Synergistic effects of granulocyte-colony stimulating factor on bone marrow stromal cell transplantation for mice cerebral infarct. *Cytokine*. 2009;46(2):260-266.
5. Chiba Y, Kuroda S, Maruichi K, et al. Transplanted bone marrow stromal cells promote axonal regeneration and improve motor function in a rat spinal cord injury model. *Neurosurgery*. 2009;64(5):991-999; discussion 999-1000.
6. Shichinohe H, Kuroda S, Lee JB, et al. In vivo tracking of bone marrow stromal cells transplanted into mice cerebral infarct by fluorescence optical imaging. *Brain Res Brain Res Protoc*. 2004;13(3):166-175.
7. Shichinohe H, Kuroda S, Yano S, Hida K, Iwasaki Y. Role of SDF-1/CXCR4 system in survival and migration of bone marrow stromal cells after transplantation into mice cerebral infarct. *Brain Res*. 2007;1183:138-147.
8. Yano S, Kuroda S, Shichinohe H, Hida K, Iwasaki Y. Do bone marrow stromal cells proliferate after transplantation into mice cerebral infarct?--a double labeling study. *Brain Res*. 2005;1065(1-2):60-67.
9. Li Y, Chopp M. Marrow stromal cell transplantation in stroke and traumatic brain injury. *Neurosci Lett*. 2009;456(3):120-123.
10. Osanai T, Kuroda S, Yasuda H, et al. Noninvasive transplantation of bone marrow stromal cells for ischemic stroke: preliminary study with a thermoreversible gelation polymer hydrogel. *Neurosurgery*. 66(6):1140-1147.
11. Chiba Y, Kuroda S, Shichinohe H, et al. Synergistic effects of bone marrow stromal cells and a Rho kinase (ROCK) inhibitor, Fasudil on axon regeneration in rat spinal cord injury. *Neuropathology*. 2009.
12. Maruichi K, Kuroda S, Chiba Y, et al. Transplanted bone marrow stromal cells improves cognitive dysfunction due to diffuse axonal injury in rats. *Neuropathology*. 2009;29(4):422-432.
13. Itosaka H, Kuroda S, Shichinohe H, et al. Fibrin matrix provides a suitable scaffold for bone

- marrow stromal cells transplanted into injured spinal cord: a novel material for CNS tissue engineering. *Neuropathology*. 2009;29(3):248-257.
14. Yasuda H, Kuroda S, Shichinohe H, Kamei S, Kawamura R, Iwasaki Y. Effect of biodegradable fibrin scaffold on survival, migration, and differentiation of transplanted bone marrow stromal cells after cortical injury in rats. *J Neurosurg*. 112(2):336-344.
 15. Shichinohe H, Kuroda S, Tsuji S, et al. Bone marrow stromal cells promote neurite extension in organotypic spinal cord slice: significance for cell transplantation therapy. *Neurorehabil Neural Repair*. 2008;22(5):447-457.
 16. Yano S, Kuroda S, Shichinohe H, et al. Bone marrow stromal cell transplantation preserves gammaaminobutyric acid receptor function in the injured spinal cord. *J Neurotrauma*. 2006;23(11):1682-1692.
 17. Yano S, Kuroda S, Lee JB, et al. In vivo fluorescence tracking of bone marrow stromal cells transplanted into a pneumatic injury model of rat spinal cord. *J Neurotrauma*. 2005;22(8):907-918.
 18. Lee JB, Kuroda S, Shichinohe H, et al. A pre-clinical assessment model of rat autogeneic bone marrow stromal cell transplantation into the central nervous system. *Brain Res Brain Res Protoc*. 2004;14(1):37-44.
 19. Bang OY, Lee JS, Lee PH, Lee G. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol*. 2005;57(6):874-882.
 20. Mendonca ML, Freitas GR, Silva SA, et al. [Safety of intra-arterial autologous bone marrow mononuclear cell transplantation for acute ischemic stroke]. *Arq Bras Cardiol*. 2006;86(1):52-55.
 21. Bliss TM, Andres RH, Steinberg GK. Optimizing the success of cell transplantation therapy for stroke. *Neurobiol Dis*. 37(2):275-283.
 22. Paul C, Samdani AF, Betz RR, Fischer I, Neuhuber B. Grafting of human bone marrow stromal cells into spinal cord injury: a comparison of delivery methods. *Spine (Phila Pa 1976)*. 2009;34(4):328-334.
 23. Jendelova P, Herynek V, DeCroos J, et al. Imaging the fate of implanted bone marrow stromal cells labeled with superparamagnetic nanoparticles. *Magn Reson Med*. 2003;50(4):767-776.
 24. Bakshi A, Hunter C, Swanger S, Lepore A, Fischer I. Minimally invasive delivery of stem cells for spinal cord injury: advantages of the lumbar puncture technique. *J Neurosurg Spine*. 2004;1(3):330-337.
 25. Vaquero J, Zurita M, Oya S, Santos M. Cell therapy using bone marrow stromal cells in chronic paraplegic rats: systemic or local administration? *Neurosci Lett*. 2006;398(1-2):129-134.

26. Borlongan CV, Chopp M, Steinberg GK, et al. Potential of stem/progenitor cells in treating stroke: the missing steps in translating cell therapy from laboratory to clinic. *Regen Med.* 2008;3(3):249-250.
27. Sugiyama T, Kuroda S, Osanai T, et al. Near-infrared fluorescence labeling allows non-invasive tracking of bone marrow stromal cells transplanted into rat infarct brain. *Neurosurgery*. In press.
28. Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski H. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*. 1986;17(3):472-476.
29. Lee J, Kuroda S, Shichinohe H, et al. Migration and differentiation of nuclear fluorescence-labeled bone marrow stromal cells after transplantation into cerebral infarct and spinal cord injury in mice. *Neuropathology*. 2003;23(3):169-180.
30. Chen J, Li Y, Wang L, et al. Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke*. 2001;32(4):1005-1011.
31. de Vasconcelos Dos Santos A, da Costa Reis J, Diaz Paredes B, et al. Therapeutic window for treatment of cortical ischemia with bone marrow-derived cells in rats. *Brain Res.* 2010;1306:149-158.
32. Komatsu K, Honmou O, Suzuki J, Houkin K, Hamada H, Kocsis JD. Therapeutic time window of mesenchymal stem cells derived from bone marrow after cerebral ischemia. *Brain Res.* 2010;1334:84-92.
33. Shen LH, Li Y, Chen J, et al. Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab.* 2007;27(1):6-13.
34. Guzman R, Choi R, Gera A, De Los Angeles A, Andres RH, Steinberg GK. Intravascular cell replacement therapy for stroke. *Neurosurg Focus*. 2008;24(3-4):E15.
35. Hicks A, Jolkkonen J. Challenges and possibilities of intravascular cell therapy in stroke. *Acta Neurobiol Exp (Wars)*. 2009;69(1):1-11.
36. Li Y, Chen J, Chen XG, et al. Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology*. 2002;59(4):514-523.
37. Chen X, Katakowski M, Li Y, et al. Human bone marrow stromal cell cultures conditioned by traumatic brain tissue extracts: growth factor production. *J Neurosci Res.* 2002;69(5):687-691.
38. Onda T, Honmou O, Harada K, Houkin K, Hamada H, Kocsis JD. Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. *J Cereb Blood Flow Metab.* 2008;28(2):329-340.
39. Toyama K, Honmou O, Harada K, et al. Therapeutic benefits of angiogenetic gene-modified human mesenchymal stem cells after cerebral ischemia. *Exp Neurol*. 2009;216(1):47-55.

40. Son BR, Marquez-Curtis LA, Kucia M, et al. Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells*. 2006;24(5):1254-1264.
41. Lundberg J, Le Blanc K, Soderman M, Andersson T, Holmin S. Endovascular transplantation of stem cells to the injured rat CNS. *Neuroradiology*. 2009;51(10):661-667.
42. Michalet X, Pinaud FF, Bentolila LA, et al. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science*. 2005;307(5709):538-544.
43. Liebert A, Wabnitz H, Obrig H, et al. Non-invasive detection of fluorescence from exogenous chromophores in the adult human brain. *Neuroimage*. 2006;31(2):600-608.
44. Steinbrink J, Liebert A, Wabnitz H, et al. Towards noninvasive molecular fluorescence imaging of the human brain. *Neurodegener Dis*. 2008;5(5):296-303.
45. Lin S, Xie X, Patel MR, et al. Quantum dot imaging for embryonic stem cells. *BMC Biotechnol*. 2007;7:67.
46. Noh YW, Lim YT, Chung BH. Noninvasive imaging of dendritic cell migration into lymph nodes using near-infrared fluorescent semiconductor nanocrystals. *FASEB J*. 2008;22(11):3908-3918.
47. The STEPS Participants. Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS): bridging basic and clinical science for cellular and neurogenic factor therapy in treating stroke. *Stroke*. 2009;40(2):510-515.

FIGURE LEGENDS

Fig. 1

Rotarod treadmill performance. Line graph shows the temporal profiles of functional recovery after permanent middle cerebral artery occlusion in direct group (red), intravenous group (blue), and vehicle group (black). The BMSC or vehicle were transplanted at 7 days after the onset of cerebral infarct. * $P < 0.05$ compared with intravenous group, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared with vehicle group.

Fig. 2

Serial *in vivo* near infrared (NIR) fluorescence imaging at 7, 14, 21, and 28 days after direct (A) or intravenous BMSC transplantation (B). The BMSC are transplanted through each delivery route at 7 days after the onset of cerebral infarct. Note that the NIR fluorescence can be clearly identified in the peri-infarct area through the skull and scalp in direct group (arrows), but not in intravenous group.

Fig. 3

Line graph shows the temporal profiles of the fluorescence intensity ratio. The values are calculated as the ratio of total efficiency in the region of interest in the right parietal region to that in the reference tube. The values are significantly higher at 14, 21, and 28 days after BMSC transplantation in direct group (red) than in intravenous group (blue). * $P < 0.05$, ** $P < 0.01$

Fig. 4

Schematic diagrams and fluorescence photomicrographs show the distributions of the QD800-positive cells at 28 days after direct (A, C) or intravenous BMSC transplantation (B, D). Note that many QD800-positive cells are distributed in the peri-infarct neocortex in direct group, but not in intravenous group (arrow). Scale bar = 100 μm .

Fig. 5

Photomicrographs of fluorescence immunohistochemistry at 28 days after direct BMSC transplantation show that some of the QD-800 positive cells are also positive for GFAP (A-C, arrow) and for NeuN (D-F, arrows) in the peri-infarct neocortex. Scale bar = 20 μm .

Fig. 1

Fig. 2

Fig. 3

Fluorescence intensity ratio

Fig. 4

Fig. 5