Cell transplantation therapy has been expected to promote functional recovery in various kinds of central nervous system (CNS) disorders, including cerebral infarct. The bone marrow stromal cells (BMSCs) may have the enormous therapeutic potential because of their capacity of neuroprotection and neural differentiation. More importantly, they can be harvested from the patients themselves without posing ethical or immunologic difficulties, and they have no tumorigenesis.1,2 Recent animal studies have demonstrated that the BMSCs significantly enhance functional recovery after ischemic stroke, when directly transplanted into the brain at clinically relevant timing.3–7 Several clinical trials have also indicated that BMSC transplantation is at least feasible for patients with CNS disorders.8 As pointed out by several investigators, however, there are several concerns to be resolved before clinical application of BMSC transplantation for CNS disorders.2,9 The issues include the development of imaging techniques to track the engrafted cells and to monitor the response of the host CNS. These techniques would enable validation of the therapeutic benefits of BMSC transplantation on the host brain in clinical situation.

123I-Iomazenil Single Photon Emission Computed Tomography Visualizes Recovery of Neuronal Integrity by Bone Marrow Stromal Cell Therapy in Rat Infarct Brain

Background and Purpose—This study was aimed to assess whether 123I-iomazenil (IMZ) single photon emission computed tomography can serially monitor the effects of bone marrow stromal cell (BMSC) transplantation on neuronal integrity in infarct brain of rats.

Methods—The BMSCs were harvested from green fluorescent protein–transgenic rats and were cultured. The rats were subjected to permanent middle cerebral artery occlusion. Their motor function was serially quantified throughout the experiments. The BMSCs or vehicle was stereotactically transplanted into the ipsilateral striatum at 7 days after the insult. Using small-animal single photon emission computed tomography/computed tomography apparatus, the 123I-IMZ uptake was serially measured at 6 and 35 days after the insult. Finally, fluorescence immunohistochemistry was performed to evaluate the distribution of engrafted cells and their phenotypes.

Results—The distribution of 123I-IMZ was markedly decreased in the ipsilateral neocortex at 6 days postischemia. The vehicle-transplanted animals did not show a significant change at 35 days postischemia. However, BMSC transplantation significantly improved the distribution of 123I-IMZ in the peri-infarct neocortex as well as motor function. The engrafted BMSCs were densely distributed around cerebral infarct, and some of them expressed neuronal nuclear antigen and γ-aminobutyric acid type-A receptor.

Conclusions—The present findings strongly suggest that the BMSCs may enhance functional recovery by improving the neuronal integrity in the peri-infarct area, when directly transplanted into the infarct brain at clinically relevant timing. 123I-IMZ single photon emission computed tomography may be a promising modality to scientifically prove the beneficial effects of BMSC transplantation on the host brain in clinical situation. (Stroke. 2013;44:2869-2874.)

Key Words: cerebral infarction ◼ iomazenil ◼ mesenchymal stromal cells ◼ tissue therapy ◼ tomography, emission-computed, single-photon
emission computed tomography (SPECT) because the central type of benzodiazepine receptor is specifically expressed in neurons.\textsuperscript{12}

Therefore, this study was aimed to evaluate whether \textsuperscript{123}I-IMZ SPECT can serially monitor the neuronal integrity and, thus, assess the beneficial effects of cell therapy in the rats subjected to cerebral infarct.

Materials and Methods

**BMSC Preparation**

All animal experiments were approved by the Animal Studies Ethical Committee of Hokkaido University Graduate School of Medicine. The BMSCs were isolated from male 8-week-old transgenic rats expressing enhanced green fluorescence protein (GFP; Japan SLC, Inc, Hamamatsu, Japan) and were cultured as previously reported.\textsuperscript{4,5,7,10} The cells were passed 3 times.

**Rat Permanent Middle Cerebral Artery Occlusion Model**

Male 8-week-old SD rats (n=23) were purchased from CLEA Japan, Inc (Tokyo, Japan). Permanent middle cerebral artery (MCA) occlusion was induced as described previously.\textsuperscript{4,5,7,10} Briefly, the rats were anesthetized, and the bilateral common carotid arteries were exposed. Then, temporal craniotomy was performed, using a small dental drill. The dura mater was kept intact, and the right MCA was ligated using 10-0 nylon thread through the dura mater. Subsequently, the bilateral common carotid arteries were occluded by surgical microclips for 1 hour.

**Transplantation of BMSCs**

The BMSCs (n=10) or vehicle (n=8) was transplanted into the ipsilateral striatum at 7 days after the onset of permanent MCA occlusion, as described previously.\textsuperscript{4,5,7,10} Briefly, the animals were fixed to a stereotactic apparatus, and the cranium was exposed through midline skin incision. A burr hole was made 3 mm right to the bregma, using a small dental drill. A Hamilton syringe was inserted 5 mm into the brain parenchyma from the surface of the dura mater, and 10 μL of cell suspension (1.0×10\textsuperscript{5} cells) or vehicle (phosphate-buffered saline) was introduced into the striatum during a period of 5 minutes, using an automatic microinjection pump.

**Behavioral Test**

Motor function of the animals was serially assessed before and at 1, 7, 14, 21, 28, 35, and 42 days after the onset of ischemia, using a Rotarod treadmill. This behavioral test was performed in all the BMSC- (n=10) and vehicle-treated rats (n=8). The Rotarod was set to the acceleration mode from 4 to 40 rpm for 3 minutes. The maximum time that the animal stayed on the Rotarod was recorded for each performance, as described previously.\textsuperscript{4,5,7,10}

\textsuperscript{123}I-IMZ SPECT

The \textsuperscript{123}I-IMZ uptake was semiquantitatively measured, using a high-resolution small-animal imaging system (Inveon SPECT/CT; Siemens Medical Solutions, Knoxville, TN).\textsuperscript{11} In this device, the SPECT and computed tomography (CT) components are combined in a common gantry, which are mounted perpendicularly. The SPECT component has dual head detector geometry that is mounted on a rotating gantry. Each detector head contains a 68×68 pixelated scintillator array of 2.0×2.0×10 mm NaI-(Tl) crystals with 0.2-mm gap, in combination with a position-sensitive photomultiplier tube readout.\textsuperscript{11}

SPECT measurements were repeated in each animal at 6 and 35 days after the onset of permanent MCA occlusion, that is, 1 day before and 28 days after stereotactic BMSC transplantation. They were held still without anesthesia, and \textsim \textasciitrangleq 80 MBq of \textsuperscript{123}I-IMZ was intravenously injected via the tail vein. They were returned to their cage and were allowed free for 60 minutes. Subsequently, they were anesthetized with 2.0% isoflurane in air and were scanned by SPECT for 90 minutes. For SPECT scan parameters, 60 projection views were acquired at 180 s/view over 360°, with single-pinhole collimator of 2.0-mm aperture, at a radius of rotation of 35 mm. The acquired data were reconstructed in the 3-dimensional ordered subset expectation maximization method with 2 iterations per 6 subsets, a voxel size of 0.5×0.5×0.5 mm\textsuperscript{3}. Neither attenuation nor scatter correction was performed. The spatial resolution for these parameters was \textsim 2.0-mm full width at half maximum. The CT images were acquired for the registration to the \textsuperscript{123}I-IMZ SPECT images. Acquisition parameters were as follows: voltage 80 kVp, anode current 500 μA, angular sampling 1° per projection for a full 360° scan, and effective pixel size 186.1 μm. Images were reconstructed using a modified Feldkamp algorithm.

Their body temperature was maintained constant between 36.5°C and 37.5°C throughout examination, using a heating pad. Round-shaped region of the interests (diameter, 1.5 mm) were symmetrically placed in the dorsal neocortex, infract core, and striatum. The ratio of ipsilateral to contralateral radioactivity was calculated, using IDL (Research Systems, Colorado) and ASIPro VM (Concorde Microsystems, Knoxville, TN).

**Immunohistochemistry**

At 5 weeks after transplantation, the animals were deeply anesthetized with 4.0% isoflurane in N2O/O2 and transcardially perfused. The brain was removed, immersed in 4% paraformaldehyde for 2 days, and embedded in paraffin. The 4-μm thick coronal sections at the levels of the striatum were prepared for subsequent analysis. Double fluorescence immunohistochemistry was performed, as previously described.\textsuperscript{4,5,7,10} Each section was treated with primary antibody against the \textgreekgamma-1 to 6 subunit of \textgreekgamma-aminobutyric acid type-A (GABA\textsubscript{A}) receptor (rabbit polyclonal, 1:50 dilution; Santa Cruz Biotechnology, Inc) or neuronal nuclear antigen (NeuN; mouse monoclonal, 1:100 dilution; Millipore) at room temperature for 1 hour and was labeled with Alexa Fluor 594 (Molecular Probes Inc, Eugene, OR) at room temperature for 1 hour. Subsequently, they were treated with primary antibody against GFP (mouse monoclonal, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) tagged with Zenon Alexa Fluor 488 (Mouse IgG Labeling Kit; Molecular Probes Inc, Eugene, OR) at room temperature for 1 hour. The fluorescence emitted was observed through appropriate filter under a fluorescence microscope and digitally photographed using a charge-coupled device camera. Two regions of the interests (450×550 μm) were symmetrically placed in the dorsal neocortex adjacent to cerebral infarct. The number of cells positive for NeuN and GABA\textsubscript{A} receptor and the ratio of ipsilateral to contralateral cell number were calculated. In the BMSC-transplanted animals, the percentages of the cells that were doubly positive cells for GFP and NeuN or GABA\textsubscript{A} receptor were also determined.

In this study, same histological analysis was performed in the rats subjected to permanent MCA occlusion at 6 days postischemia as the controls (n=5) to evaluate histological findings at same time as the initial SPECT examination.

**Statistical Analysis**

All data were expressed as mean±SD. Continuous data were compared by unpaired \textit{t} test. Values of \textit{P}<0.05 were considered statistically significant.

**Results**

**Effects of BMSC Transplantation on Functional Recovery**

As shown in Figure 1, all animals exhibited severe neurological deficit during 1 week after the onset of focal cerebral ischemia. There was no significant difference in motor function between the vehicle- and BMSC-treated animals. Subsequently, the vehicle-transplanted animals did not show
any significant improvement of motor function. However, motor function in the BMSC-transplanted animals significantly improved at 4 and 5 weeks after BMSC transplantation ($P<0.05$; Figure 1).

**Effects of BMSC Transplantation on Neuronal Integrity**

Using SPECT, the $^{123}$I-IMZ uptake was semiquantitatively measured at 6 and 35 days postischemia. As shown in Figure 2, visual observations revealed a marked decrease in the distribution of $^{123}$I-IMZ in the ipsilateral neocortex at 6 days postischemia. In the vehicle-transplanted animals, the distribution of $^{123}$I-IMZ did not change in the peri-infarct neocortex at 35 days postischemia. However, BMSC transplantation improved the distribution of $^{123}$I-IMZ in the peri-infarct neocortex at the same timing.

In the vehicle-treated animals, the ipsilateral-to-contralateral ratios of radioactivity in the peri-infarct neocortex were 58.6±24.8% and 59.5±20.1% at 6 and 35 days after ischemia, respectively. There was no significant difference between 2 values ($P=0.2665$; Figure 3). However, the value significantly increased from 53.4±17.3% to 77.3±16.2% in the BMSC-treated animals ($P<0.01$). The ratio was significantly higher in the BMSC-treated animals than in the vehicle-treated animals at 35 days postischemia ($P<0.05$). These findings were not observed in the striatum and infarct core (Figure 3).

**Histological Analysis**

Figure 4 shows histological findings in the ipsilateral and contralateral neocortex adjacent to cerebral infarct. Hematoxylin and eosin staining revealed that a significant number of neurons was damaged in the ipsilateral neocortex adjacent to cerebral infarct in the controls. The ratio of the ipsilateral to contralateral number of NeuN-positive cells was 79.9±7.3% at 6 days postischemia. The values were 78.2±16.9% in the vehicle-treated animals at 42 days postischemia, suggesting that the processes of neuronal damage were completed in the peri-infarct neocortex by 6 days postischemia. However, the value was 89.9±10.8% in the BMSC-treated animals at 42 days postischemia, that is, at 35 days post-transplantation. Their value was significantly higher than those in the controls at 6 days and in the vehicle-treated animals at 42 days postischemia ($P<0.05$).

Likewise, the ratio of the ipsilateral to contralateral number of GABA$_A$ receptor-positive cells was 75.4±3.6% in the controls at 6 days postischemia. The values were 74.2±15.3%, and 90.1±9.5% in the vehicle- and BMSC-treated animals at 42 days postischemia, respectively (Figure 5). The value was significantly higher in the BMSC-treated animals than in the controls ($P<0.01$) and in the vehicle-treated animals ($P<0.05$).

Double fluorescence immunohistochemistry revealed that the GFP-positive cells (26.4±14.8/region of the interests) were widely distributed in the peri-infarct neocortex. Some of them were also positive for NeuN (15.7±7.5%) or GABA$_A$ receptor (7.6±4.7%; Figure 6).

**Discussion**

Using a small-animal SPECT/CT apparatus, this study serially visualizes the effects of BMSC transplantation on the distribution of $^{123}$I-IMZ in infarct brain of the living rodents.
As a result, the engrafted BMSCs improve neuronal integrity in the peri-infarct area and enhance functional recovery after ischemic stroke. The BMSCs are densely distributed in the peri-infarct area, and some of them express the neuronal phenotype. Thus, 123I-IMZ SPECT may be a promising modality to assess the therapeutic benefits of cell therapy for ischemic stroke without subjective bias.

As aforementioned, 123I-IMZ is a ligand displaying high affinity for central-type benzodiazepine receptors. The benzodiazepine receptor is a part of the postsynaptic GABA receptor complex and presents in high concentration on all intact cortical neurons. According to previous studies, 123I-IMZ is known as a useful tracer to assess neuronal viability in various kinds of CNS disorders such as Alzheimer's disease, epilepsy, and ischemic stroke. Animal experiments have also shown that 123I-IMZ is a useful marker of neuronal viability on autoradiography. Thus, Kuge et al. reported that 123I-IMZ uptake markedly decreased in the infarct regions at 4 and 24 hours after the onset of MCA occlusion. Kaji et al. also showed that neuronal DNA was still intact in the ischemic regions where 123I-IMZ uptake was preserved. Using autoradiography, we have previously shown that the engrafted BMSCs express the marker protein specific for GABAA receptor and significantly improve the distribution of 125I-IMZ in the peri-infarct area. Similar results have been obtained in the rat model of spinal cord injury. However, autoradiography allows observation at only 1 time point by postmortem study and cannot serially evaluate brain function in the living rodents. Furthermore, previous SPECT scanners could not assess it in the small animals because of their limited spatial resolution. Therefore, to the best of our knowledge, this is the first study that demonstrates the usefulness of 123I-IMZ SPECT to evaluate the therapeutic effects of cell therapy on neuronal integrity in each living rodent longitudinally and noninvasively, using the small-animal SPECT/CT system with excellent spatial resolution.

Very recently, we serially assessed local glucose metabolism in the rats subjected to permanent MCA occlusion, and found that BMSC transplantation significantly enhances the recovery in the peri-infarct area, using small-animal 18F-fluorodeoxyglucose positron emission tomography/CT system. Thus, glucose use was markedly decreased in the ipsilateral neocortex at 6 days after ischemia. In the vehicle-treated...
animals, glucose use improved to some extent in the peri-infarct neocortex at 35 days after ischemia. However, BMSC transplantation significantly enhanced the recovery in the peri-infarct neocortex at the same time point. Considering together with the present results, the BMSCs may enhance the recovery of local glucose metabolism by improving neuronal integrity in the peri-infarct area, when directly transplanted into the infarct brain because oxidative glucose metabolism is quite high in the neurons. Therefore, BMSC transplantation may possibly contribute to accelerate functional recovery by improving neuronal integrity and local metabolism in the peri-infarct brain. However, an alternative possibility is not completely excluded. The increase in FDG uptake seen on 35 days after middle cerebral artery occlusion in rat might indicate a simple reflection of macrophage activity or gliosis. Histological findings support the speculation. Thus, a certain subgroup of neurons is selectively damaged in the peri-infarct neocortex on both hematoxylin and eosin staining and immunostaining at 6 days postischemia. The findings do not change in the vehicle-treated animals thereafter. However, the density of NeuN- and GABA<sub>α</sub>-receptor–positive cells significantly increase in the BMSC-treated animals at 42 days postischemia. Furthermore, the GFP-positive cells were widely distributed in the peri-infarct neocortex. A part of them were also positive for NeuN or GABA<sub>α</sub> receptor, suggesting their neural differentiation. Previous studies have shown that the engrafted BMSCs may enhance functional recovery after ischemic stroke through multiple mechanisms. Some of them have the potential to replace the injured tissue by differentiating into the neural cells. Another subgroup possibly releases the neuroprotective or neurotrophic factors and supports the survival of damaged neurons. Alternatively, they may enhance neurogenesis in the host CNS. Therefore, it is most likely that the engrafted BMSCs may improve the neuronal integrity in the peri-infarct area by their multiple biological activities, including neuronal differentiation by themselves and enhanced neurogenesis in the host brain. In addition, we previously showed that some of transplanted BMSC also express the astrocytic phenotype in the corpus callosum in addition to neuronal markers such as NeuN and microtubule-associated protein-2. Furthermore, our recent study has suggested that the engrafted BMSCs also protect the neurovascular integrity between basement membrane and astrocyte end-feet and ameliorate brain damage in stroke-prone spontaneous hypertensive rats.

As described above, it would be essential to bridge the still existing gap between preclinical studies and clinical investigations to achieve clinical application of cell therapy for ischemic stroke. Based on the history of preclinical studies for neuroprotective drugs, noninvasive imaging technique may provide biologically relevant end point, although functional outcome was only end point in previous clinical testing of cell therapy. From this viewpoint, ¹²³I-IMZ SPECT may contribute to establish cell therapy as a scientifically proven therapy entity by serially and noninvasively validating the effects of cell therapy on the host CNS.

Conclusions

The present findings strongly suggest that the BMSCs may enhance functional recovery by improving the neuronal integrity in the peri-infarct area, when directly transplanted into the infarct brain at clinically relevant timing. ¹²³I-IMZ SPECT may be a promising modality to scientifically prove the beneficial effects of BMSC transplantation on the host brain in clinical situation.

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Disclosures

None.

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